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Interaction between Chronic Stress and Pain: the HPA axis and potential hypothalamic-medulla-spinal pathways involved

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Professor Doutor Armando Almeida
e do
Professor Doutor Nuno Sousa

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DECLARAÇÃO

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Á Gigi e ao Kiko

"By learning you will teach,

by teaching you will understand"

(Latin proverb)

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Abstract

Pain is an essential tool for survival, but equally important is the ability to overcome pain in life threatening events, allowing us to react. Stress-induced analgesia exemplifies this concept as a strong acute stressor activates the hypothalamic-pituitary-adrenal glands (HPA) axis and leads to the release of adrenaline, dampening pain processing and resulting in transient analgesia. This initial response is followed by the release of corticosteroids in order to re-establish physiological homeostasis. On the other hand, the effects of chronic stress upon pain perception are not consensual as both inhibition (analgesia) and facilitation (hyperalgesia) of nociception has been reported in different contexts. Additionally, several studies showed that patients suffering from chronic pain display altered HPA axis function, suggesting an intricate relationship between the stress and pain modulatory systems.

To assess the effect of chronic stress upon nociception, a chronic unpredictable stress (CUS) paradigm was used in order to warranty a strong and sustained corticosteroid release throughout the experimental period. The specific contribution of each corticosteroid receptor towards spinal nociception was also evaluated by mimicking CUS through prolonged administration of corticosterone [mineralocorticoid- (MR) and glucocorticoid-receptor (GR) agonist] and dexamethasone (GR agonist alone), while the influence of CUS upon the supraspinal pain control system was assessed by behavioural and electrophysiological approaches. The tract-tracing anatomical identification of the pain modulatory areas targeted by the paraventricular nucleus of the hypothalamus (PVN), the coordinator of the stress response, set the basis for the electrophysiological evaluation of a direct role for the PVN upon descending antinociception in a normal and chronic pain (arthritis) scenario. In parallel, the role in pain modulation of the dorsomedial nucleus of the hypothalamus (DMH), a contributor to stress-induced hyperalgesia, was also evaluated in normal and arthritic rats using the same behavioural and electrophysiological approaches.

Our results show that the unpredictability of stressor application is essential for a strong HPA axis activation and subsequent behavioural analgesia. The spinal antinociceptive effect of sustained corticosteroid levels appears to be mediated by GR receptor activation, through the decreased expression of nociceptive neurotransmitters and enhanced availability of $GABA_{B2}$ receptors. At

the supraspinal level, CUS decreased both the number and the spontaneous and noxious-evoked activity of rostral ventromedial medulla (RVM) pronociceptive ON-cells, thus indicating a role for the RVM in chronic stress-induced analgesia. The anatomical analysis of the PVN brain connections confirmed that this nucleus is part of an intricate bi-directional neuronal network that comprises several supraspinal areas involved in pain modulation, amongst which are the RVM and caudal ventromedial medulla (CVLM). The pharmacological manipulation of the PVN demonstrated that it has an antinociceptive role, mediated by descending serotonergic and noradrenergic pathways (but not opioidergic). The serotonergic PVN-mediated effects were depressed following the development of arthritis, which suggests that plastic changes in the pain control system should contribute to chronic nociceptive pain. Additionally, we demonstrated that the PVN has a tonic and phasic descending antinociceptive effect upon nociceptive modulation, which is partly mediated through the RVM and CVLM. Interestingly, hyperalgesia induced by stress shares at least a common denominator with antinociceptive descending modulatory pathways, as RVM nociceptive cells are also modulated by the DMH. The manipulation of the DMH demonstrated its facilitatory nociceptive effect in normal animals. However, the DMH is not involved in the pain-induced hyperalgesia occurring during chronic nociceptive pain, as its nociceptive facilitatory drive is absent in arthritic animals. Finally, the descending drive from the PVN, but not the DMH, is maintained in the presence of arthritis, which points to a stronger involvement of the HPA axis in chronic inflammatory disorders.

In conclusion, analgesia induced by CUS is the reflex of profound changes in the neurochemistry of the spinal dorsal horn and descending pain modulatory pathways. In chronic inflammation, the HPA axis enhanced activity could contribute to the comorbidities associated with pain due to the detrimental effects of corticosteroid hypersecretion that occur during its activation. Future studies should evaluate the role of limbic (emotional and cognitive) areas, like the amygdala, upon CUS and the PVN, and the potential alterations in these areas triggered by the development of chronic inflammatory pain. This will elucidate on the mechanisms of emotional alterations associated with chronic stress and chronic pain.

Resumo

A capacidade de percepcionar estímulos dolorosos é uma ferramenta essencial para a sobrevivência de um indíviduo, apenas ultrapassada pela capacidade de conseguir inibir a dor em situações de risco nas quais é necessário reagir para sobreviver. A analgesia induzida pelo stress é um exemplo deste mecanismo uma vez que numa situação stressante aguda, capaz de activar o eixo hipotálamo-hipófise-suprarrenais (HPA), a imediata secreção de adrenalina interfere com a transmissão nociceptiva e resulta numa analgesia intensa mas temporária. Posteriormente e como resultado da activação do eixo HPA, ocorre a libertação de corticosteróides cuja função é re-estabelecer a homeostasia fisiológica. Por outro lado, o efeito do stress crónico na percepção dolorosa não é consensual uma vez que, dependendo do contexto experimental, foi já descrita tanto uma diminuição da dor (analgesia) como o seu incremento (hiperalgesia). Para além disto, diversos estudos clínicos demonstram que pacientes com dor crónica sofrem de disfunção do eixo HPA, o que realça a existência de uma interacção importante e complexa entre os mecanismos que modulam a resposta à dor e ao stress.

Na avaliação do efeito do stress crónico sobre a nocicepção usou-se um paradigma de stress crónico imprevisível (CUS) por forma a garantir uma secrecção sustentada de corticosteróides ao longo de todo o período experimental. A contribuição específica de cada um dos receptores de corticosteróides na modulação espinhal da nocicepção foi avaliada através da administração prolongada de corticosterona [agonista dos receptores mineralocorticóides (MR) e glucocorticóides (GR)] e de dexametasona (agonista específico de receptores GR), que mimetizaram fisiologicamente os efeitos do CUS. A influência do CUS sobre o sistema supraspinhal de controlo da dor foi avaliada em termos comportamentais e pela caracterização electrofisiológica da actividade neuronal destas áreas.

A posterior identificação, através de traçadores anatómicos retrógrados e anterogrados, de áreas moduladoras da dor que recebem projecções por parte do núcleo paraventricular do hipotálamo (PVN), a área coordenadora da resposta fisiológica ao stress, serviu de base para a avaliação

electrofisiológica do envolvimento directo deste núcleo na modulação descendente da dor em animais normais ou com dor crónica (artrítica). Em paralelo, procedeu-se à avaliação comportamental e electrofisiológica do papel do núcleo dorsomedial do hipotálamo (DMH) na modulação da dor, já que este núcleo parece mediar a hiperalgesia induzida pelo stress.

Os nossos resultados demonstram que a aleatoriedade na aplicação prolongada do stress é um factor determinante na sustentação da activação do eixo HPA e na subsequente analgesia. O efeito antinociceptivo do stress crónico ao nível da medula espinhal parece ser mediado pela activação de receptores GR, os quais diminuem a expressão de neurotransmissores nociceptivos e aumentam a disponibilidade dos receptores $GABA_{B2}$ no corno dorsal da medula espinhal. A nível supraspinhal, o CUS induziu uma diminuição do número, uma diminuição da actividade espontânea e uma diminuição da resposta a estímulos nóxicos das células pronociceptivas ON do bolbo rostroventromedial (RVM), evidenciando a participação desta área na analgesia induzida pelo stress crónico.

A avaliação anatómica das projecções do PVN confirmou que este núcleo faz parte de uma extensa rede neuronal que compreende conexões recíprocas com várias áreas encefálicas envolvidas na modulação da dor, como sejam o RVM e o bolbo caudoventrolateral (CVLM). Adicionalmente, a manipulação farmacológica do PVN demonstrou que esta área exerce uma acção antinociceptiva através de vias descendentes serotonérgicas e noradrenérgicas (mas não opioidérgicas). O efeito antinociceptivo serotonérgico do PVN está deprimido após o desenvolvimento de artrite, o que sugere a ocorrência de alterações plásticas no sistema de controlo da dor que deverão contribuir para a dor crónica nociceptive. A acção antinociceptiva tónica e fásica do PVN sobre a transmissão nociceptiva espinhal, é parcialmente mediada pelo RVM e CVLM. Curiosamente, a hiperalgesia induzida pelo DMH partilha parcialmente as vias anatómicas com o sistema antinociceptivo descendente da dor, uma vez que as células do RVM também são moduladas pelo DMH. A manipulação do DMH confirmou o seu papel pronociceptivo em animais normais. No entanto, o DMH não participa na hiperalgesia induzida pela dor crónica pois a acção facilitatora deste núcleo desaparece nos animais artríticos. Mais ainda, a acção moduladora do PVN (mas não do DMH) é mantida na presença de artrite, o que sugere um maior envolvimento do eixo HPA nas doenças inflamatórias crónicas.

Em conclusão, a analgesia induzida pelo CUS é o espelho de alterações profundas na neuroquímica da medula espinhal e das vias descendentes intervenientes no sistema endógeno

supraspinhal de controlo da dor. Em patologias inflamatórias crónicas, o aumento da actividade do eixo HPA poderá contribuir para as comorbidades associadas à dor como consequência dos efeitos deletérios da hipersecreção prolongada de corticosteróides que daí resultam. Estudos futuros poderão avaliar o papel das áreas do sistema límbico (emocional-cognitivo), como a amigdala, sobre o CUS e o PVN e as alterações plásticas nestas mesmas áreas resultantes do desenvolvimento de dor crónica inflamatória. Os dados resultantes contribuirão para elucidar os mecanismos das alterações emocionais associados ao stress crónico e à dor crónica.

Abbreviations List

AMY – Amygdala

ARTH – Arthritis/arthritic

BNST – Bed Nucleus of the Stria Terminalis

CGRP - Calcitonin gene-related peptide

CORT – Corticosterone

CRF – Corticotrophin releasing factor

CUS - Chronic unpredictable stress paradigm

CVLM - Caudal ventrolateral medulla

DEX – Dexamethasone

DMH - Dorsomedial hypothalamic nucleus

DRt - Dorsal reticular nucleus (DRt)

GDNF - Glial cell line-derived neurotrophic factor

GR - Glucocorticoid receptors

HPA axis - Hypothalamus-pituitary-adrenals axis (HPA).

LC – Locus coeruleus

MPO – Median preoptic nucleus

MR - Mineralocorticoid receptors

NGF – Nerve growth factor

NS - Nociceptive-specific neurones

NTS – Nucleus tractus solitarius

PAG - Periqueductal gray matter

PFC – Prefrontal cortex

PVN - Paraventricular nucleus of the hypothalamus

RA – Rheumatoid arthritis

RVM - Rostral ventromedial medulla

SIA - Stress-induced analgesia

SIH – Stress-induced hyperalgesia

SOM – Somatostatin

SP - Substance P

WDR - Wide-dynamic-range neurones

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Chapter One

Introduction

1. INTRODUCTION

1.1. Pain as a defence mechanism

Pain is an aversive experience that results in learned avoidance behaviours, which protect the organism to potentially threatening noxious events. Its perception results from the processing of the emotional and sensitive components usually evoked by a painful stimulus and classically attributed to the activation of the medial and lateral pathways (respectively) of the spinothalamic tract (Hodge and Apkarian, 1990). The International Association for the Study of Pain (IASP) defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Apkarian, 2008). It is a broadspectrum definition that seeks to encircle all the dimensions of a subjective experience like pain and of special importance since pain was not considered a disease until the fourth quarter of the last century. The subjectivity of pain results from the fact that, when faced with the same stimulus, each individual perceives pain differently and the amount of tissue damage might not be directly proportional to pain intensity (Ossipov, 2009). Additionally, pain may chronify and remain for long periods, even though no pathological cause is associated with it or when the original lesion is already healed, as a result of the deregulation of pain mechanisms (Apkarian, 2008).

The most common type of pain is acute or protective pain. Acute pain reflects the encoding of a noxious stimulus from the periphery to the central nervous system by specific fibres, the nociceptors (Basbaum and Jessell, 2000). Acute pain is a short-lived sensation ranging from seconds to minutes in duration and, most importantly, it ceases at the same time, or soon after, the noxious stimulus does (Melzack 1999; Millan, 1999). The myriad of afferent sensory modality inputs is the result of interplay between the myelinisation degree of transmitting fibres, the type of peripheral receptors and the neurotransmitters expressed by each fibre and spinal cord targets. Of the three fibre types (Aβ, Aδ and C-fibres) of primary afferent neurones involved in the transmission of sensory information, only Aδ and C-fibres are involved in acute nociceptive transmission (D'Mello and Dickensen, 2008). However, it has been recently argued that the overall exclusion of Aβ-fibres from nociceptive processing is mainly the consequence of

experimental limitations (Djouhri and Lawson, 2004). Additionally, Aβ-fibres may be involved in pain arising during chronic pain syndromes (Lekan *et al.*, 1996).

1.1.1. Nociceptors

Aδ- and C-fibres, responsible for fast and slow nociceptive transmission (respectively) (Meyer *et al.*, 2008), contribute differently to the development and maintenance of pain (Pertovaara, 1998; Fuchs *et al.*, 2000, Magerl *et al.*, 2001). Besides the velocity of transmission, these fibres can be further subdivided into groups according to the type of noxious stimulation to which they respond (mechanical, thermal or chemical stimulation) (Lewin and Moshourab, 2004), with a fibre responding to more than one modality being denominated as polymodal (Magerl *et al.*, 2001). Large and some small myelinated fibres terminate in the dermis ensheathed by Schwann cells, forming encapsulated endings that are involved in innocuous perception (Meisnner, Merkel and Vater-Pacini corpuscles), whereas Aδ and C-fibres terminate as free-nerve endings in the dermis and the epidermis (Willis, 2007).

Non-myelinated C-fibres can be divided in peptidergic and nonpeptidergic fibres according to its neurotransmitter expression (Snider and McMahon, 1998). Peptidergic C-nociceptors express both calcitonin gene-related peptide (CGRP) and substance P (SP) and project to lamina I and outer lamina II (IIo) of the spinal cord dorsal horn, whereas non-peptidergic C-fibres project only to inner lamina II (IIi) (Coutaux *et al.*, 2005). The third group of nociceptors comprise the thinly-myelinated Aδ-neurones, with fibres terminating in lamina I; a set of Aδ-fibres terminate also in lamina V upon spinal nociceptive neurones receiving convergent input from Aδ- (nociceptive) and Aβ- (innocuous) fibres (multireceptive neurones; see below) (Basbaum *et al.*, 2009). Aδ-nociceptors contain CGRP and SP and their membrane express receptors for nerve growth factor (NGF) (McCarthy and Lawson, 1989; Coutaux *et al.*, 2005). On the other hand, non-peptidergic C-nociceptors express the ATP-gated ion channel P2X3 and express c-Ret neurotrophin receptors that respond to glial-derived neurotrophic factor (GDNF) (Snider and McMahon, 1998; Hunt and Mantyh, 2001; Zylka *et al.*, 2005; Zylka, 2005) whereas peptidergic C-neurones express TrkA neurotrophin receptor targets of NGF (Coutaux et al., 2005; Basbaum *et al.*, 2009). A part of the nociceptors containing CGRP also expresses somatostatin (SOM) (Sakamoto *et al.* 1999). It is worth noting that while spinal CGRP originates exclusively from primary afferents (Chung *et al.*,

1988), SP is also present in some spinal neurons and in axons descending from the brainstem (Todd and Spike, 1993; Ribeiro-da-Silva and Hokfelt, 2000).

1.1.2. Spinal Cord and Ascending Pathways

Intense stimulation (noxious) of peripheral receptors is transduced by membrane receptors in an electrical signal that is conveyed by C- and Aδ-fibres and result in the release of neurotransmitters in the dorsal horn of the spinal cord (Millan, 1999). Inputs from peripheral nociceptive are distributed at different lamina levels of the dorsal horn, and although most nociceptors synapse in laminae I and II, some projection neurones in laminae IV-V are also targeted (Sugiura *et al.* 1986). Central branches of primary afferent axons terminating in the spinal dorsal cord are orderly distributed based on the degree of myelinisation, sensory modality and location of the receptive field (Todd, 2002). As shown above, while C-fibres mainly synapse in lamina I and II neurones (Sugiura *et al.*, 1986), Aδ-afferents project to laminae II and V (Light & Perl, 1979; Craig *et al.*, 1988) and large-diameter Aβ-fibres terminate on deep laminae III to V (Brown *et al.*, 1981). The transmission to and from the spinal cord to supraspinal centres is not a linear process, as the arriving nociceptive signal may be transmitted supraspinally ipsi- or contralaterally, and it may undergo either local modular processing by spinal interneurones (Lu and Perl, 2005) or supraspinal descending modulation (Ren and Dubner, 2002) (see below).

The spinal targets of peripheral sensory fibres are nociceptive-specific (NS), wide-dynamic-range (WDR) and non-nociceptive neurones (NNS) (Han *et al.*, 1998). NS or high-threshold neurones, which respond merely to noxious stimulation, include projecting neurones and interneurones and are found mainly in lamina I, while WDR (multireceptive cells), which respond both to noxious and non-noxious stimulation and are found predominantly in the deep dorsal horn (Menetrey *et al.*, 1977). Low-threshold NNS cells respond exclusively to non-noxious stimulation (Willis and Coggeshall 1991). Nociceptive spinofugal neurones send their axons through ascending spinal tracts and convey information on the discriminative and emotional aspects of pain to supraspinal centres for processing the multidimensional pain experience (Braz *et al.*, 2005; Lima, 2009; Polgár *et al.*, 2010) (**Figure 1**).

One of the most important ascending pathways is the spinothalamic tract (STT). This pathway comprises a lateral part that projects principally to the ventral posterolateral and ventral posteromedial nuclei of the thalamus (VPL and VPM) and conveys information related to the

sensory-discriminative aspects of pain, and a medial part projecting to the medial thalamus and related to the autonomic and emotional aspects of pain (Albe-Fessard *et al.*, 1985; Lima, 2009). The VPL is mostly targeted by WDR neurones originating from spinal laminae I and V and relays nociceptive information to the somatosensory cortices S1 and S2 (Shi and Apkarian, 1995; Price 2002). The medial pathway projects to the medial thalamus and, through a spinofugal pathway relaying in the parabrachial nuclei (PB), the bed nucleus of the stria terminalis (BNST) (Bester *et al.*, 2000), the amygdala (AMY), hypothalamus, nucleus cuneiformis, the periaqueductal gray matter (PAG) (Hylden *et al.*, 1989) and the anterior cingulated cortex (ACC) (Bernard and Besson, 1990; Bernard and Bandler, 1998). Other spinoreticular projections included in the medial pain system are those spinal connections with the medullary dorsal reticular nucleus (DRt) (Almeida *et al.*, 1993; 1995) and the caudal ventrolateral medulla (CVLM) (Lima and Coimbra, 1991).

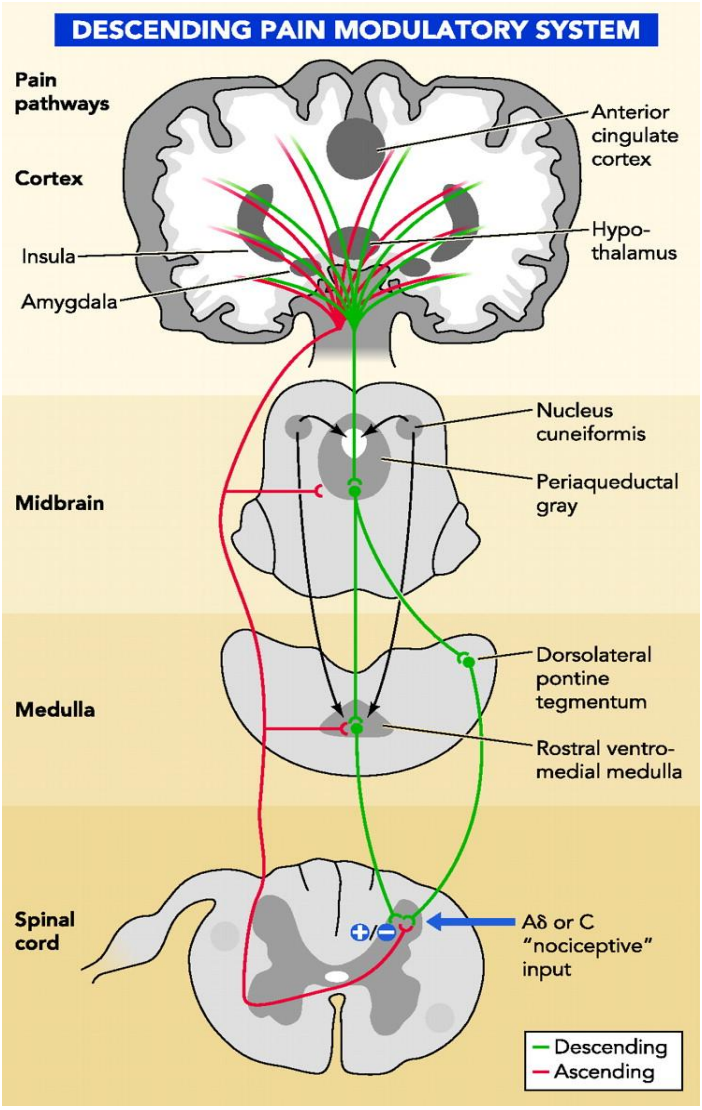

Figure 1 – Schematic representation of the neuronal networks involved in nociceptive processing (Bingel and Tracey, 2008).

1.1.3. Supaspinal pain processing

Human brain imaging studies helped to identify the multiple forebrain regions activated during acute painful stimulation (Apkarian, 1995). The main brain regions that are activated during an acute painful experience include the primary and secondary somatosensory cortex, the anterior

cingulated and prefrontal cortices and the thalamus (Tracey, 2007). Other regions as the basal ganglia, cerebellum, AMY and the hippocampus can also be activated depending on the experimental designs (Tracey and Bushnell, 2009). Recently, the insular cortex and prefrontal cortex (PFC) have been associated to the discrimination of pain intensity (Oshiro *et al.*, 2009) while the posterior parietal cortex and right dorsolateral PFC are more involved in spatial discrimination of pain (Oshiro *et al.*, 2009). The ACC is also important for the processing of pain unpleasantness and learned avoidance behaviours (Craig *et al.* 1996; Apkarian and Shi 1998; Malin *et al.*, 2007). Human imaging studies have demonstrated the activation of the ACC after noxious (Casey, 1999), but not innocuous, stimulation (Hutchinson *et al.*, 1999). ACC glutamatergic projections have been shown to participate in pain facilitation through direct projections to the DRt (Lima and Almeida, 2002; Zhang *et al.*, 2005).

The AMY receives nociceptive information from lamina I and IIo neurones (Jasmin *et al.* 1997) via the spino-PB-amygdaloid pathway. Information received by the lateral and basolateral amygdaloid nuclei (Bernard and Besson 1990; Bernard *et al.* 1996) and relayed to the central nucleus of the amygdala (CeA), an area involved in the affective and autonomic responses to pain (Bernard *et al.*, 1996; Neugebauer and Li, 2003; Ansah *et al.*, 2009). The AMY is involved in analgesia by opioids, an effect mediated by PAG (Pavlovic and Bodnar 1998) and the rostral ventromedial medulla (RVM) (Helmstetter *et al.*, 1998; McGaraughty *et al.*, 2004). Moreover, synaptic plasticity in the AMY has been shown following the development of arthritis (Neugebauer *et al.*, 2003), whereas structural alterations and plasticity of both nociceptive AMY neurones (Gonçalves *et al.*, 2008) and their descending modulatory action upon RVM cells (Ansah *et al.*, 2009) were demonstrated in neuropathic pain.

Another target of the spino-PB-amygdaloid pathway is the hypothalamus (Bernard *et al.*, 1993; Bester *et al.*, 1997) (**Figure 1**), an important structure in the regulation of neuroendocrine and autonomic functions, strongly involved in homeostasis during nociception (Saadé and Jabbur, 2008). A number of nuclei within the hypothalamus have already been associated with pain processing: the ventromedial hypothalamus is implicated in innate affective reaction to pain (Borszcz, 2006), particularly in fear-induced antinociception (Freitas *et al.*, 2009); the anterior hypothalamic area differentially modulates C- and A-nociceptive inputs to the spinal dorsal horn (Simpson *et al.*, 2008); the lateral hypothalamic area (Holstege, 2004) projects to the RVM and produces analgesia when activated (Holden and Pizzi, 2008); in the suprachiasmatic nucleus,

the oxytocin expression decreased after noxious stimulation (Yang *et al.*, 2008a); the supraoptic nucleus is an antinociceptive nucleus (Yang *et al.*, 2008b) that projects to the brainstem, namely to the PAG, RVM and locus coeruleus (LC) (Sawchenko and Swanson, 1982); the arcuate nucleus is related to antinociception as it is implicated in the release of endogenous opioids (Sun and Yu, 2005) and has serotonergic and noradrenergic projections to the PAG, RVM, LC, PB, and nucleus tractus solitarius (NTS) (Sim and Joseph, 1991); finally, the medial preoptic area (MPO) is involved in autonomic and sensory functions through its projections to the PAG and reciprocal serotonergic projections to the NRM (Murphy *et al.*, 1999) and has a role in supraspinal action of prostaglandins (Heinricher *et al.*, 2004). Most of these areas project densely to other hypothalamic nuclei as well as to areas involved in pain descending modulation (Millan, 1999; Parry *et al.*, 2002). Two of the main hypothalamic areas implicated in descending pain modulation, specifically in stress-induced analgesia (SIA) and stress-induced hyperalgesia (SIH), the paraventricular nucleus of the hypothalamus (PVN) and the dorsomedial hypothalamic nucleus (DMH), respectively, as discussed below.

1.1.4. Pain descending modulatory systems

As evidenced above, the modulation of pain is not restricted to a single pathway but a multitude of systems, facilitatory and inhibitory, working in parallel. Classically, two areas were initially associated with antinociception, the descending pain modulatory PAG-RVM-spinal cord pathway (Basbaum and Fields, 1984; Fields *et al.*, 1991) (**Figure 1**). Later, other areas were also involved in the positive and negative signalling of pain by descending pathways targeting the spinal dorsal horn (Almeida *et al.*, 2006), amongst these being the DRt (Lima and Almeida, 2002) and the CVLM (Tavares and Lima, 2002).

The PAG comprises distinct functional areas differentially involved in emotional coping and strongly interconnected (Jansen *et al.*, 1998). Although being considered for a long time a powerful antinociceptive area (Basbaum and Fields, 1984), it has now been shown that PAG activation enhances the activity of A-fibres while simultaneously inhibiting C-fibre information (Waters and Lumb, 2008), thus being able of both an antinociceptive and pronociceptive descending modulatory action. Afferent input to the PAG arrives from forebrain limbic areas as well as structures in the brainstem (Shipley *et al.*, 1991). The PAG shares reciprocal connections

with the PVN, the MPO, the AMY, the PFC and insular cortex (Herrero *et al.*, 1991; Shipley *et al.*, 1991). It receives also a strong afferent input from the spinal cord (Behbehani, 1995), but spinal projections are sparse (Mouton and Holstege, 1994). Importantly, it targets the RVM (Li *et al.*, 1990; Hudson and Lumb, 1996) and strongly projects to the CVLM (Cobos *et al.*, 2003) and the DRt (Almeida *et al.*, 2002).

The RVM is considered the output centre of the midline pain-modulation system (PAG-RVM-spinal dorsal horn) since it mediates the descending modulatory effects of different forebrain areas (see above). The RVM projects to both superficial and deep dorsal horn laminae (Fields *et al.*, 1995). It is a nucleus defined by function rather than anatomical limits, encompassing the raphe magnus nucleus and adjacent reticular formation. Electrophysiological studies on the RVM revealed the existence of three different cell types (Fields and Heinricher, 1985), based on their firing pattern upon peripheral noxious stimulation. The ON-cells, neurones that start firing immediately before a motor response to noxious stimulation occurs, have been associated with the facilitation of pain. In contrast, the OFF-cells, which stop firing immediately before a motor response to noxious stimulation occurs, are considered antinociceptive (Neubert *et al.*, 2004). A third cell type was identified, the NEUTRAL-cells, although no changes in activity were recorded prior or during noxious stimulation. The RVM was the first area where facilitation of nociception was observed, and a major area where the duality/balance of pain descending facilitation/inhibition has been demonstrated (Heinricher *et al.*, 2009). Afferent input to this area arises from several nuclei within the cortex, the AMY, the hypothalamus, the PAG and the PB (Herman *et al.*, 1997). Efferent RVM projections follow a topographical organization and end mainly in the spinal cord dorsal horn (Mason, 2001) with ON- and OFF-cells projecting to laminae I, II and V (Fields *et al.*, 1995), those spinal areas containing the Aδ- and C-fibre central terminations and spinal nociceptive neurones (see above).

The DRt was the first exclusively nociceptive facilitatory nucleus described (Almeida *et al.*, 1996; 1999). The DRt is part of a reciprocal excitatory loop with the spinal cord that is activated by cutaneous and visceral noxious stimulation (Villanueva *et al.*, 1988**;** Almeida and Lima, 1997; Lima and Almeida, 2002; Almeida *et al.*, 2006). Accordingly, Sotgiu and colleagues (2008) demonstrated that this nucleus is involved in the maintenance of spinal sensitization in neuropathic pain states. It also shares reciprocal projections with other brainstem nuclei as the RVM, LC, CVLM, NTS, PAG and with forebrain structures like the AMY and PVN (Almeida *et al.*,

2002; Leite-Almeida *et al.*, 2006), all of which are involved in descending pain modulation. In addition to its direct pronociceptive role upon homeotopic noxious stimulation, it decreases neuronal background activity thus enhancing the relevance of the threatening stimulation (Bouhassira *et al.*, 1992).

The CVLM has been shown to have antinociceptive properties, not only by producing profound analgesia after electrical stimulation (Gebhart and Ossipov 1986; Janss and Gebhart 1987), but also by tonically inhibiting spinal nociceptive neurones (Mansikka *et al.*, 1996; Tavares *et al.*, 1997). Tavares and Lima (2002) postulated that the CVLM modulated nociceptive transmission through direct and indirect pathways to the spinal dorsal horn. Moreover, it was also proposed that the CVLM descending modulation mechanism appears to be specific to the nature of the stimuli and the type of cell where it originates from (Tavares and Lima, 2002). It has been demonstrated that the CVLM has reciprocal connections with lamina I and II spinal neurones (Tavares and Lima 2002) and with the RVM and the NTS (Cravo and Morrison, 1993; Kawano and Masuko, 1996; Cobos *et al.*, 2003). Another interesting fact is that the CVLM, as part of the ventrolateral medulla (VLM), is strongly involved in the integration of autonomic functions (Ally, 1998), namely the hypoalgesia resulting from increased blood pressure (Tavares *et al.*, 1997b). Additionally, a noradrenergic nociceptive modulation from the CVLM was shown to be mediated by an indirect descending action relaying in the noradrenergic A5 group (Tavares *et al.*, 1997a; Marques-Lopes *et al.*, 2010). Finally, the CVLM and the NTS are part of a three-way loop that includes supraspinal reciprocal connections with the PVN (Krukoff *et al.*, 1994; Kawano and Masuko, 1996).

Overall, nociception arriving at the spinal dorsal horn by peripheral sensitive fibres is conveyed by ascending tracts to several interconnected cerebral structures involved in sensorimotor functions and emotional processing. Information resulting from these areas is transmitted to brainstem structures directly involved in pain modulation through descending projections to the spinal dorsal horn. The resulting spinal modulation in normal conditions is translated into a direct presynaptic modulation of primary afferents terminals and/or postsynaptic modulation of both second order spinofugal projection neurones and spinal interneurones (Fields *et al.*, 1991). In pathological conditions, the ability to process and control nociception is compromised, the areas involved in the pain circuitry suffer plastic changes leading to the disruption between

antinociceptive and pronociceptive actions (Vanegas and Schaible, 2004; Almeida *et al.*, 2006) and the resulting pain chronification no longer has a valuable biological function.

1.2. Chronic pain

Persistent pain is a debilitating factor for both the physiological and psychological health of patients (Bair *et al.*, 2003; Gambassi, 2009) and, contrarily to acute pain, it has no apparent survival value. The central nervous system is a dynamic structure and its plasticity constitutes the basis for the development of pathological conditions (Apkarian *et al.*, 2009), even though the cellular and molecular mechanisms behind it are not completely clear. The common clinical signs that accompany the development of chronic pain (Dworkin *et al.*, 2007; Apkarian *et al.*, 2009) are hyperalgesia, allodynia and spontaneous pain (Baron, 2009; Schaible *et al.*, 2009), although differences between neuropathic and nociceptive persistent pain in man have been reported. By definition, hyperalgesia is the exacerbation of pain after a painful stimulus, allodynia is the sensation of pain after an innocuous stimulus is applied and spontaneous pain refers to pain sensation that arises without any sort of stimulation (Neumann *et al.*, 1996). All these chronic pain symptoms are a consequence not only of an increased responsiveness from nociceptors (Lumpkin and Caterina, 2007) but also of plasticity of both central nociceptive neurones and the peripheral Aβ-fibres that normally signal innocuous sensation (Devor, 2006; 2009). In chronic pain, neuropathic pain is the result of nerve injury or disease, in either the peripheral or central nervous system, while nociceptive pain (like in arthritis) is a consequence of the prolonged activation of nociceptors due to injury and is usually accompanied by inflammation (Basbaum and Jessel, 2000).

1.2.1. Pain in arthritis

A major public health problem that decreases life quality with heavy costs on national health systems is arthritis, or inflammation of the joints. Arthritis is one of the major causes of disability in adults, in the USA it affects more than 21% of the adults (46 million) but it is expected to increase to 40% by 2030 (Helmick *et al.*, 2008). The incidence of arthritis is slightly higher in women and 66% of the patients are under 65 years old. Within arthritis large spectrum, osteoarthritis represents about 60% of the cases with the hands and knees been the most affected parts. Rheumatoid arthritis (RA) constitutes 3% of the cases with higher incidence in older people (Lawrence *et al.*, 2008). In terms of the economical impact of the disease, one in

four general practice consultations in the UK are associated with arthritis. In the USA, direct and indirect costs of arthritis sum up to $86.2 billion ($51.1 billion and $35.1 billion, respectively). In the UK, estimated costs of treating arthritis are £5.7 billion/year besides being the second most common cause for absenteeism (206 million working days), corresponding to production loses of £18 billion. 90% of referrals for physiotherapy are arthritis related, costing £1.6 billion in community and social services (Helmick *et al.*, 2008).

In addition to postganglionic sympathetic fibres present in surrounding areas, three types of primary fibres innervate the knee joints, Aβ-, Aδ- and C-fibres (Schaible *et al.*, 2009). Aβ-fibres are mostly involved in proprioception and are activated by innocuous stimulation, while Aβ and C-fibres are mainly high threshold fibres that respond to noxious mechanical stimulation (nociceptors) (McDougall, 2006; Schaible *et al.*, 2009). In arthritis, inflammation causes the release of prostaglandins, histamine, cytokines, bradikinins amongst other inflammatory molecules, either by the peripheral termination of primary afferents, tissue cells or immune system cells (D'Mello and Dickensen, 2008), from the injured site to the adjacent areas. Nociceptors in the primary damage areas and later in the surrounding areas suffer plastic changes in response properties due to their permanent activation by inflammatory signals (Schaible *et al.*, 1991). In monoarthritis of the knee joint, per example, former high-threshold mechanically insensitive fibres become responsive after inflammation and are thought to be at the origin of joint pain (Schaible et al., 2009). Inflammation caused increased firing rates of these fibres during normal joint movements and even during rest periods as often reported by arthritic patients. A large proportion of articular neurones are peptidergic with a majority expressing SP, CGRP and SOM (Schaible *et al.*, 2009), with SP and CGRP displaying increased expression (Buma *et al.*, 2000) following arthritis. In addition to their role in vasodilatation and vascular permeability in neurogenic inflammation, SP is involved in peripheral sensitization of mechanical receptors (Heppelmann and Pawlak, 1997) while CGRP is correlated to the hypersensitivity of spinal neurones during the development of inflammatory states, as well as in its maintenance (Neugebauer *et al.*, 1996). Peripheral sensitization of primary afferents is accompanied by central sensitization and is thought to set the basis for the development of chronic pain syndromes. Central sensitization, or the excitability enhancement of second-order neurons in the spinal cord and brain, is at the origin of the hyperalgesia and allodynia (Schaible *et al.*, 2002) that, if prolonged in time, leads to the reorganization of pain modulatory pathways (McDougall, 2006). fMRI studies on patients suffering from knee osteoarthritis revealed a bilateral

enhancement of brain activity in the thalamus, S2, insular, and cingulate cortices and unilateral increased activity in the putamen and AMY (Baliki *et al.*, 2008).

During the development of arthritis, an increase in the net descending nociceptive facilitation is initially observed (Urban and Gebhart, 1999), but the continuous peripheral inflammation gradually leads to enhancement of spinal descending nociceptive inhibition (Ren and Dubner, 1996). Pinto and colleagues (2007) demonstrated a time dependent shift in *c-fos* evoked expression between the spinal cord and brainstem modulatory centres during inflammatory pain development, attributed to changes in the circuitry mediating this process (Almeida *et al.*, 2006). These dynamic time-dependent changes have been associated with the activation/deactivation of the inhibitory and the facilitatory descending pain modulatory systems within the RVM and LC (Ren and Dubner, 1996; Wei *et al.*, 1999; Ren and Dubner, 2002). Early inflammatory descending facilitation was correlated to dorsal horn hyperexcitability resulting from the release of excitatory amino acids in the spinal cord (Heinricher *et al.*, 1999). Additionally, Miki and colleagues (2002) observed after a few hours of inflammation not only a phenotype shift of RVM cells from neutral to ON- and OFF-cells, but also changes in the baseline activity of these cells (Ren and Dubner, 2002).

Comorbidity between arthritis and depression and anxiety has been reported in chronic pain patients (McWilliams *et al.*, 2004; 2008). Interestingly, Neugebauer and Li (2003) observed an increase in the firing rate of AMY neurones (CeA) after the noxious mechanical stimulation of inflamed joints. The AMY is involved in the affective and autonomic responses to pain (Neugebauer and Li, 2003) and is able to modulate RVM neuronal activity, inhibiting or facilitating nociception (McGaraughty and Heinricher, 2002). In chronic pain, it has been demonstrated that this pathway undergoes plastic changes (Ansah *et al.*, 2009), which are paralleled by structural changes in the AMY (Gonçalves *et al.*, 2008). Another study, in patients suffering from primary hip osteoarthritis, that investigated possible morphologic alterations in brain areas involved in pain transmission, reported a gray matter decrease in the anterior cingulate cortex (ACC), the right insular cortex and operculum, the dorsolateral prefrontal cortex (DLPFC), the AMY and the brainstem (Rodriguez-Raecke *et al.*, 2009). Importantly, these changes were partly reverted once pain was effectively treated (Rodriguez-Raecke *et al.*, 2009).

In summary, inflammatory diseases are a major cause of disability on working adults resulting in direct and indirect costs that have a huge economical impact on society. In arthritis, the increase

in primary afferent input, due to direct stimulation or indirect sensitization of nociceptive and non-nociceptive fibres, leads to central sensitization and compromises normal sensorimotor and emotional processing. Profound modulatory adjustments, involving both pain facilitation and inhibition, are primarily observed in brainstem areas involved in descending nociceptive modulation, probably as a result of changes in forebrain areas of the limbic (emotional and cognitive) system that modulate pain through the brainstem.

1.2.2. The HPA axis, glucocorticoids and arthritis

If we could dissect arthritis in two separate components, inflammation and pain, signalling pathways for both would share a common player, the hypothalamus-pituitary-adrenals axis (HPA). Inflammation activates the HPA, as part of the endogenous anti-inflammatory pathways and noxious signalling, and triggers a cascade of autonomic, endocrine and immune responses involved in the defensive behavioural response (Coutinho and Chapman, 2010). In normal conditions, HPA activation leads to the release of the corticotrophin-releasing hormone (CRH) and vasopressin (AVP) by the PVN into the hypophyseal portal vasculature (**Figure 2**). The binding of CRH to its receptors in the pituitary induces the secretion of adrenocorticotropic hormone (ACTH), which promotes the release by the adrenal glands of cortisol in humans or corticosterone (CORT) in rodents, potent endogenous anti-inflammatory agents, to the systemic circulation. In normal conditions, the HPA is modulated by a negative feedback loop in which CORT will inhibit the production of CRH by the PVN and stop its response. In some chronic inflammatory diseases, like RA, both humans and animals display deficiencies in HPA axis function that results in a decreased production of corticosteroids (Sternberg and Wilder, 1989; Wilder, 1996; Morand and Leech, 2001; Cutolo *et al.*, 2002). HPA impairment leads to decreased production of CRH and CORT, which in turn have been shown to enhance arthritis susceptibility (Sternberg *et al.*, 1989), a positive feedback mechanism thought to contribute to the maintenance of intractable pain (Tracey, 2002). Indeed, recent data shows that the administration of prednisone, a synthetic corticosteroid, reduces morning stiffness and pain in RA patients (Kirwan *et al.*, 2010).

Later studies in patients with RA also verified that HPA hyporesponsiveness was not restricted to the hypothalamus but also to blunt responsiveness of adrenal glands (Dekkers *et al.*, 2001). Also as a result of inflammation, a third element can be added to this plot - mood disorders and plastic alterations of the frontal-amygdalar circuits behind it (Marques, *et al.*, 2009). Not only is

the AMY capable of directly modulate pain, but other areas of the limbic system such as hippocampus and medial prefrontal (Herman *et al.*, 2005) are able to mediate the activity of the HPA (Lupien *et al.*, 2009). The AMY stimulates hypothalamic CRF release through serotonergic and adrenergic receptors in the parvocellular PVN (Feldman and Weidenfeld, 1998), an effect that is enhanced by elevated circulating CORT levels (Shepard *et al.*, 2003). Chronic pain states, mood disorders and persistent inflammation seem to function as positive feedback mechanisms, as they mutually exacerbate each other.

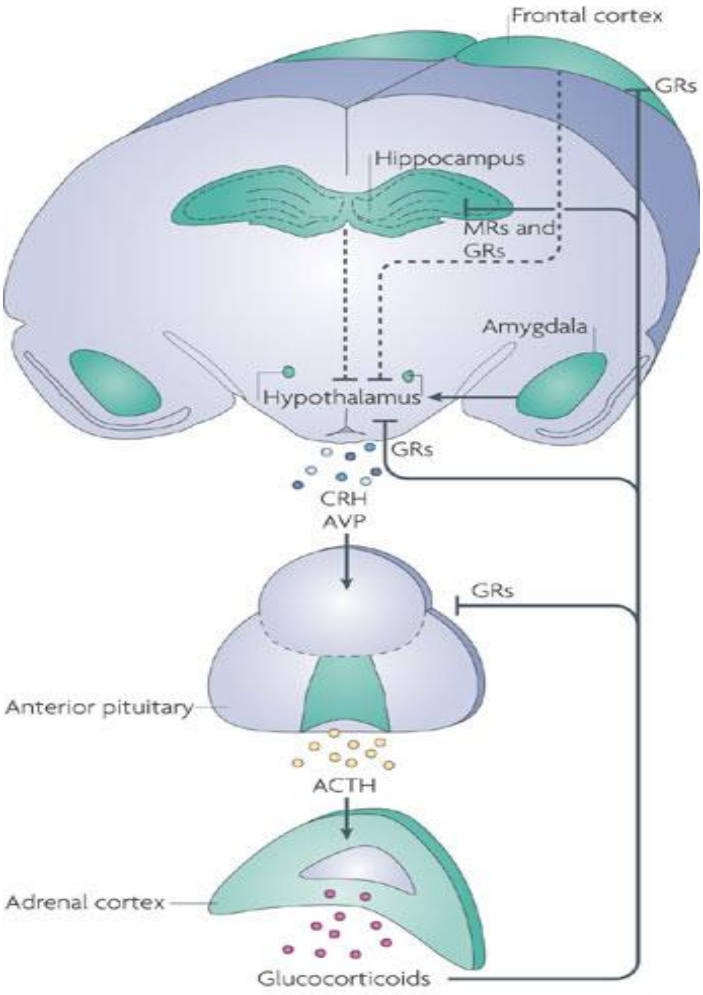

Figure 2 – Schematic representation of the stress circuitry (Lupien *et al.*, 2009).

In response to inflammation, the production of CRH increased in order to raise circulating levels of cortisol (Webster and Sternberg, 2004). The negative feedback mechanism adjusting cortisol/corticosterone production is mediated by glucocorticoid binding to its receptors either directly in the hypothalamus and pituitary (Webster and Sternberg, 2004) or indirectly through nuclei within the hippocampal formation, prefrontal cortex, septum and medial preoptic area (Ziegler and Herman, 2002; McEwen, 2007; Cerqueira *et al.*, 2008). Corticosteroids bind, with

different affinities (Reul *et al.*, 1987), to two receptors, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (Reul and De Kloet, 1985). GR expression varies amongst species although its distribution is similar. In primates and rodents, GR receptors are expressed mainly in the hippocampal CA1 and CA2, dentate gyrus, amygdala, PFC, cerebellum, PVN and cortical layers II/III and VI, whereas MR receptors can be found in the hippocampus, AMY, PFC, caudate-putamen, lateral septum and cortical layers II/III and V (Ahima and Harlan, 1990; Ahima *et al.*, 1991; Patel *et al*, 2000). Receptor binding affinity is physiologically important as low CORT affinity for GR is considered to constitute the basis for the negative feedback mechanism of glucocorticoids, while high MR affinity has been associated to the maintenance of CORT levels and HPA homeostasis (Ratka *et al.,* 1989; Pace and Spencer 2005; Sousa *et al.*, 2008). Over the last decades, the use of glucocorticoids in the treatment of rheumatoid arthritis as an anti-inflammatory agent has consistently increased (Buttgereit *et al.*, 2004), although prolonged usage causes well-characterized clinical side-effects (Rauchhaus *et al.*, 2009). While corticosteroid administration is primarily targeted at controlling inflammation levels, a direct inhibitory action on pain perception (antinociceptive) has also been described (Habib *et al.*, 2010). One hypothesis is that increased circulating levels of cortisol alter the ability to retrieve consolidated memories therefore decreasing the emotional component of on-going pain (Quervain *et al.*, 2009), as observed in some anxiety disorders. On the other hand, glucocorticoids have been shown to directly modulate SP, CGRP and SOM content in dorsal root ganglia (Smith *et al.*, 1991). The spinal dorsal horn is also a major site of GR expression (Gonzalez *et al.*, 1990; Marlier *et al.*, 1995), which has been shown to be enhanced after peripheral nerve injury (Yan *et al.*, 1999).

1.3. Pain modulation by the hypothalamus

The perpetuation of pain and inflammation is strongly associated with the HPA and, therefore, to hypothalamic function. Both factors can be considered as discrete stressors in which temporal prolongation produces mal-adaptive responses that are not present in normal conditions. As with acute pain, an acute stress response represents an important protective mechanism for the survival of the individual. Under circumstances of great stress or fear, like in life threatening events, acute intense stress produces profound analgesia, a phenomenon known as SIA (Ford and Finn, 2008; Butler and Finn, 2009). A positive correlation between the analgesia and the activation of the ipsilateral ACC and the AMY has been shown (Zhang *et al.*, 2003), an effect that

is mediated by connections with the PAG (Helmstetter, 1993; Floyd *et al.*, 2000; Zhang *et al.*, 2003) and RVM (Ford and Finn, 2009). Hypothalamic-mediated SIA is thought to be processed either by direct projections to the spinal cord (Robinson *et al.*, 2002; Rojas-Piloni *et al.*, 2007) or indirectly through efferent projections to the PAG and the RVM (Cechetto and Saper, 1988), which would in turn inhibit nociceptive transmission at the spinal cord (Butler and Finn, 2009). Lesion as well as stimulation studies of several hypothalamic nuclei have shown that each subnuclei differently mediates SIA (Butler and Finn, 2009) and several neurotransmitters expressed in the hypothalamus have also being associated with SIA, amongst which are vasopressin (Wideman *et al.*, 1996), oxytocin (Robinson *et al.*, 2002), neurotensin (Seta *et al.*, 2001) and orexin (Watanabe *et al.*, 2005). Concerning the PVN, its lesion did not alter the analgesic response of animals to restraint stress (Fuchs and Melzack, 1996), but the involvement of corticosteroids in SIA was demonstrated by Filaretov and colleagues (1996) when the administration of DEX in the PVN reduced SIA. However, the role of CORT and CRF in SIA is still a matter of discussion as both factors are not only able to promote opioid release but also have anti-inflammatory actions known to alter nociception (Ford and Finn, 2008).

Not all forms of acute stress decrease nociception, as anxiety has been proven to increase sensitivity to pain (Rhudy and Meagher, 2001), an event also known as stress-induced hyperalgesia (SIH) (Imbe *et al.*, 2006). Unlike fear, anxiety arises from uncertain expectation associated with negative anticipation of an uncontrollable event (Ford and Finn, 2008). In humans, decreasing anxiety levels of chronic pain patients reduces several components of pain, such as the intensity and unpleasantness of a stimulus (Rhudy and Meagher, 2001). Albeit the numerous animal and human studies, the mechanisms involved in SIH are still not clear and several pathways at both the supraspinal and the spinal cord levels have been proposed. Animal models of SIH involve the repeated exposure of animals to inescapable non-noxious stressful events like serial defeat stress (Marcinkiewcz *et al.*, 2009), cold environments (Satoh *et al.*, 1992), periods of restraint (Silva-Torres *et al.*, 2003) or forced swimming (Bradesi *et al.*, 2005), followed by an acute measure of thermal, chemical and/or mechanical nociception. Although mainly associated with analgesia, a pronociceptive role has been attributed to the PAG under stressful conditions, which involves cholecystokinin (CCK) (Lovick, 2008) and its antagonist action upon opioid analgesia (Tortorici *et al.*, 2003). Alternatively, Quintero and colleagues (2000) proposed that SIH after repeated stress was the result of a decrease in central serotonin activity. Also important is the activation of the HPA, as the release of glucocorticoids and

catecholamines modify signalling pathways in nociceptors, which enhance hyperalgesia evoked by inflammatory molecules like prostaglandins and bradykinin (Khasar *et al.*, 2005; Khasar *et al.*, 2008). Concerning the spinal cord, repeated swimming increased basal c-fos expression of lumbar laminae I, II, V, and VI (Quintero *et al.*, 2003) and cold stress enhanced spinal SP and CGRP. A role for the HPA axis in SIH is thus reinforced since SP and CGRP co-localize and are regulated by glucocorticoid receptors in the spinal cord (DeLeo *et al.*, 1994). Moreover, Suarez-Killian and colleagues (1995) observed that stress decreased $GABA_A$ receptor activation in the spinal cord, a place where presynaptic inhibition classically occurs. The decrease in GABA receptor expression could then increase the PVN descending oxytocinergic pathway mediated by spinal GABAergic interneurones upon WDR nociceptive neurones (Rojas-Piloni *et al.*, 2007), thus enhancing spinal nociceptive transmission (desinhibition).

Stress dependent analgesia or hyperalgesia is strongly mediated by emotions and appear to be dependent on prefrontal cortex activity (Neugebauer *et al.*, 2009). Fear and anxiety seem to be at the basis of the nociceptive outcome since when faced with an uncontrollable event; anticipation will increase pain perception, while unpredictability will originate analgesia.

1.3.1. The paraventricular nucleus of the hypothalamus (PVN)

The PVN plays a pivotal role in the modulation of the HPA axis and is a major integration centre of several circuits as body homeostasis (Blair *et al.*, 1996), circadian rhythm (Pickard & Turek, 1983), food intake (Leibowitz *et al.*, 1990), gastrointestinal and cardiovascular functions (Li *et al.*, 2006), learning and memory (McEwen, 2004). Although in the human brain a division between parvocellular and magnocellular parts cannot be established as different areas overlap (Goudsmit *et al.*, 1992), in rats PVN neurones can be divided in five parvocellular (dorsal, lateral, medial periventricular and anterior subnuclei) and three magnocellular (anterior, posterior and medial magnocellular subnuclei) areas (Goufman, 1991). PVN neurones can also be divided into three groups according to their function: (1) neurones that secrete factors involved in neurohypophysis function (produce vasopressin and oxytocin); (2) neurones involved in autonomic control, and (3) neurones involved in modulating HPA axis activity (Herman *et al.*, 2008). This was confirmed by a recent study by Geerling and colleagues (2010).

Forebrain input to the PVN varies slightly between species, in the rat it originates mainly from the lateral septal nucleus and the subicular cortex, the circumventricular organs, the subfornical

organ, the amygdala (Silverman *et al.*, 1981) and the paraventricular thalamic nucleus (Ferguson *et al.*, 1984). Within the hypothalamus, major afferent projections originate from the preoptic, suprachiasmatic, ventromedial and arcuate nuclei, the retrochiasmatic and lateral hypothalamic areas, and the contralateral PVN (Silverman *et al.*, 1981). Along the rostro-caudal neuraxis the density of afferent projections decreases (Larsen *et al.*, 1991), the main caudal areas projecting to the PVN being the PB, LC, ventral medulla, A1 catecholamine cell group and NTS (McKellar and Loewy, 1981).

Neurones in the PVN project densely to other hypothalamic areas, such as the arcuate, the dorsomedial, the ventral premammillary nuclei and the preoptic region (Wittman *et al.*, 2009). Forebrain areas targeted by the PVN include the subfornical organ and organum vasculosum laminae terminalis (Larsen *et al.*, 1991), the tuber cinereum area, the central amygdaloid nucleus, the bed nucleus of the stria terminalis, the lateral septal nucleus, the paraventricular thalamic nucleus and median eminence (Carsen *et al.*, 1990; Wittman *et al.*, 2009). More caudal targets are the median eminence, the PAG (Larsen *et al.*, 1991), the NTS (van der Kooy *et al.*, 1984), the DRt (Almeida *et al.*, 2002), the ventrolateral medulla (Pyner and Coote, 1999), including the CVLM (Cobos *et al.*, 2003) and the spinal cord (Sawchenko and Swanson, 1982). The contribution of the PVN to pain modulation under stress conditions seems obvious (see above). However, a direct involved of the PVN in the descending modulation of pain was only established when its electric or chemical stimulation induced profound analgesia (Shiraishi *et al.*, 1995), whereas its lesion decreased nociceptive threshold (Yang *et al.*, 2008c). To the best of our knowledge, a systematic evaluation of both the efferent and afferent connections of the PVN with areas of the supraspinal pain control system has still not been performed in a single study. As the intricate architecture of the PVN is accompanied by a complex neuropeptide secretion pattern (McEwen, 2004), numerous studies have been performed in an attempt to discriminate neurotransmitters and pathways that mediate this effect.

Vasopressin (AVP) is expressed by both the magnocellular and parvocellular part (Swanson and Sawchenko, 1983) and about 40% of the cells in the medial and dorsal parvocellular subdivisions project to the spinal cord and several brainstem areas (Hallbeck and Blomqvist, 1999). However, Yirmiya and colleagues (1990) verified that direct PVN-AVP projections were not involved in nociception and it has since been proposed that PVN-AVP induced analgesia would result from multisynaptic projections to the spinal cord that involved relays in the PAG (Yang *et al.*, 2006b),

the RVM (Yang *et al.*, 2006c) and the caudate nucleus (Yang *et al.*, 2006a). In addition, AVP-PVN projections to the PAG have been shown to terminate upon cells expressing enkephalin, endorphin and dynorphin (Yang *et al.*, 2007), the classic mediators of opioid analgesia (Basbaum and Fields, 1984).

About 11 to 16% of PVN neurones projecting to the spinal cord and located in the lateral and medial parvocellular subdivision (Sawchenko and Swanson, 1982; Hallbeck *et al.*, 2001), express oxytocin (OT), a neurotransmitter also involved in spinal nociceptive processing (Condes-Lara *et al.*, 2006). PVN-OT projections have been proposed to be activated (Condés-Lara *et al.*, 2008) and to inhibit evoked input from Aδ and C afferents to the superficial spinal cord (Condés-Lara *et al.*, 2006), through the activation of GABAergic interneurones (Breton *et al.*, 2008) in the substantia gelatinosa (spinal lamina II) (Condés-Lara *et al.*, 2009a;2009b). Curiously, an identical effect was also observed in deeper spinal laminae, but only in neuropathic animals (Condés-Lara *et al.*, 2003; 2005). The firing pattern of superficial and deeper laminae cells differs as wind-up of deeper spinal neurones, which does not occur in superficial neurones, could be prevented by OT inhibition of the NMDA receptor (Condés-Lara *et al.*, 2003) in healthy subjects. Importantly, the electric stimulation of the PVN resulted in the increase of OT levels not only in the spinal cord but also in the cerebrospinal fluid (CSF) and plasma (Martinez-Lorenzana *et al.*, 2008), and thus the possibility of the involvement of other players cannot be discarded.

PVN neurones expressing GABA are predominantly involved in the modulation of the sympathetic outflow (Zhang and Patel, 1998). GABAergic neurones predominate in the dorsal and ventral parts of the PVN and surrounding the dorsal cap (Pyner, 2009). PVN GABAergic projections to the spinal cord have been proposed to modulate presynaptically the depolarization of incoming Aδ and C-fibers (Condes-Lara *et al.*, 2008). In this context, GABAergic interneurones present in lamina I and II (Heinke *et al.*, 2004) that colocalize oxytocin receptors (Reiter *et al.*, 1994) would inhibit primary afferent input (Rojas-Piloni *et al.*, 2007).

The PVN is a main local of CRF synthesis, and CRF is strongly involved in the mechanisms mediating acute as well as chronic pain. The distribution pattern of its receptors (Van Pett *et al.*, 2000) in association with the CRF effect on the activation of the HPA axis and its pronounced effect in clinical pain has lead to its consideration as a possible pain modulator (Lariviere and

Melzack, 2000). However, the role of CRH in nociception is not consensual, as early works reported both analgesia and hyperalgesia (Bunnett, 2005; Vit *et al*, 2006) or no effect (Ayesta and Nikolarakis, 1989). The mechanisms of CRF analgesia are also not clear (Mousa *et al.*, 2007) as some studies stated that it is not mediated by opioids, as spinal opioid antagonists did not alter pain threshold after intracerebroventricular administration of CRH (Yarushkina, 2008). However, CRF in inflamed tissue is required for opioid receptor-specific antinociception (Schafer *et al.*, 1996). Inflammatory pain induced an upregulation of CRF receptors, which was concomitant with increased levels of enkephalin expression in interneurones in spinal lamina II (Mousa *et al.*, 2007). Additionally, CRF inhibits nociceptive behavior when injected in the AMY (Cui *et al.*, 2004), it activates noradrenergic pathways in the LC (Lejeune and Millan, 2003) and it stimulates OT release by the PVN (Robinson *et al.*, 2002). On the other hand, Hummel and colleagues (2010) recently revealed a higher degree of complexity on the role of CRF in nociception, as CRF_1 receptors are implicated in hyperalgesia.

A role for epinephrine in PVN-induced analgesia has recently been proposed (Pertovaara, 2006). Palkovits and colleagues (1999) observed an increase in the release of cathecolamines in PVN during sustained pain stimulation, and the activation of α_2-adrenoreceptors (α_2-ADR) decreases GABAergic inhibition of PVN spinal-projecting neurones (Li *et al.*, 2005). Interestingly, α_2-ADR expression is significantly increased in arthritis (Brandt and Livingston, 1990) and is thought to be involved in the inhibition of hypersensitivity (Mansikka *et al.*, 2004).

1.3.2. The dorsomedial nucleus of the hypothalamus (DMH)

The DMH mediates sympathetic regulation of cardiovascular activity in emotional stress. Either its activation or desinhibition mimetizes changes in arterial pressure, heart rate (Hunt *et al.*, 2010) as well as neuroendocrine and behavioural changes that are observed after acute emotional stress (Dimicco *et al.*, 2002). It is also involved in food consumption (Renner *et al.*, 2010), thermoregulation (DiMicco and Zaretsky, 2007), regulation of ACTH levels (Bailey and Dimicco, 2001) as well as CORT (Bernardis and Bellinger, 1987) and cathecolamine (Wible *et al.*, 1988) secretion and in the regulation of circadian cycles (Bellinger *et al.*, 1976). It has been proposed that the DMH shares functions with the PVN in the coordination of the physiological, behavioural, and neuroendocrine responses evoked by stress and emotion (DiMicco *et al.*, 2002).

In an extensive anatomical study, Thompson and Swanson (1998) observed that most DMH inputs also arise from within the hypothalamus (preoptic, arcuate, anterior hypothalamic, suprachiasmatic, posterior periventricular and dorsal tuberomammillary nuclei and the parvocellular PVN), whereas afferents from other forebrain areas include the thalamus, the ventral subiculum, infralimbic PFC, lateral septal nucleus and BNST. Brainstem afferent areas include the PAG, PB, and ventrolateral medulla. Concerning DMH efferents, the most densely hypothalamic areas innervated by the DMH are the PVN, the periventricular zone, the preoptic, suprachiasmatic and parastrial nuclei and the retrochiasmatic area. Descending projections target primarily the PAG, the pontine reticular formation, the NTS (Thompson *et al.*, 1996) and the RVM (Nogueira *et al.*, 2000); contrary to what is observed for the PVN, almost no efferents are observed in the spinal cord.

The application of a series of stressors, like footshock, restraint, swimming and immune stimulation, resulted in increased *c-fos* expression in the DMH is observed after (Elmquist *et al.*, 1996). At least some aspects of the circuits underlying stress appear to be regulated by GABAergic inhibition in both the DMH and PVN and the extensive interconnections between each other (Thompson and Swanson, 1998; Singru *et al.*, 2005). The blockade of $GABA_A$ receptors or the excitation of DMH neurones projecting to the PVN-projecting neurones activates CRF- and AVP-releasing neurons and, consequently, the HPA axis (Keim and Shekhar, 1996). On the other hand, it has been shown that DMH desinhibition causes thermal hyperalgesia through the activation of ON-cells in the RVM (Martensen *et al.*, 2009). Moreover, this study also demonstrated a link between behavioural SIH and DMH disinhibition, as a consistent increase of RVM ON- and a decrease of RVM OFF-cell firing rate was observed.

1.4. Aims

The hypothalamus stands as a privilege brain area in the integration of the physiological and psychological responses to stress. However, it is a neurochemical mosaic and part of an intricate and extensive positive and negative feedback neuronal network involved in many vital functions. Although it is involved in nociception, some aspects appear to be more pronounced in the presence of several pathologies. Additionally, chronic stress can induce antinociception or pronociception and the role of the hypothalamus in these processes is not fully understood.

The experimental design in this work focused on the specific components of the stress response that contribute to the modulation of pain. More specifically, we aimed at:

1. Analysing the influence of chronic stress upon pain-like behaviour through the use of a chronic unpredictable stress (CUS) paradigm (Chapter 2.1.1) and the chronic exogenous administration of corticosteroid receptor agonists (Chapter 2.1.2);

2. Evaluating the effect of CUS upon the activity of nociceptive neurones in a mapor supraspinal pain control centre, the RVM (Chapter 2.1.3);

3. Identifying anatomical supraspinal targets of the PVN as potential sites for stress-induced nociceptive modulation (Chapter 2.2.1);

4. Evaluating the role of the RVM (Chapter 2.3.1) and CVLM (Chapter 2.3.2) as relay centres of the descending nociceptive modulatory action of the PVN, in normal and arthritic animals;

5. Assessing whether SIA and SIH share common descending pathways within the pain modulating circuitry through the analysis the role of the RVM in the nociceptive modulatory action from the DMH, in normal and arthritic animals (Chapter 2.3.3).

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Chapter 2

Experimental work

Chapter 2.1

Chronic stress and nociceptive modulation

Chapter 2.1.1

Pinto-Ribeiro F, Almeida A, Pêgo JM, Cerqueira J, Sousa N.

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Chronic unpredictable stress inhibits nociception in male rats

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Abstract

Chronic stress elicits remarkable alterations to the structure and function of several areas of the central nervous system. Nociception is known to be affected by chronic stress and age, although the observations are contradictory. Herein we report that both young and old rats submitted to a chronic unpredictable stress paradigm have reduced nociception in the tail-flick nociceptive test. Moreover, stressed animals show an increase in nociceptive threshold after three successive exposures to noxious stimulation (within a 2 min interval). While the sustained stress-induced analgesia is usually attributed to the resulting hypercorticalism, the immediate exacerbation of tolerance to pain displayed by stressed animals is most likely mediated through other mechanisms due to the very rapid antinociceptive effect observed.

Keywords: Rat; Chronic unpredictable stress; Pain tolerance; Tail-flick; Antinociception

Tissue injury and inflammation are associated with persistent stimulation of nociceptors, which transmit the nociceptive input from the periphery to second-order neurons located in the spinal cord dorsal horn [12]. The transmission of nociceptive messages at the spinal level can be modulated by the supraspinal pain control system through endogenous mechanisms mediated by descending projections to the spinal dorsal horn [8,12]. Recent studies have shown that these mechanisms involve the descending facilitation or inhibition of spinal nociceptive transmission triggered by the activation of supraspinal *pronociceptive* or *antinociceptive* areas [9,12], respectively. Thus, it is probable that pain may result from the balance between opposing supraspinal modulatory actions impinging upon dorsal horn neurons mediating the transmission of nociceptive information to the brain [12].

Nociception can also be modulated by external factors through alterations in intrinsic pain pathways. Exposure to acute stress is amongst these factors, as it is known to produce analgesia [4]. Yet, nociceptive modulation following prolonged exposure to stress is still a matter of dispute, with some studies reporting an increased pain threshold [1] while others demonstrate hyperalgesia [4]. While the mechanisms involved in these phenomena remain cryptic, it is well known that prolonged exposure to stressful stimuli activates the hypothalamo-pituitary-adrenocortical axis (HPA), resulting in sustained elevations of corticosterone (the dominant endogenous glucocorticoid in rodents) [6].

During their lives organisms are challenged with various stressors and the ability to handle them varies considerably with age [15]. There is substantial evidence that aging impacts on neuroendocrine stress responses, and also in their deleterious consequences upon brain structure and function [15]. In addition, age differences in the sensitivity to phasic nociceptive stimulation have also been reported but show contradictory results: there are studies demonstrating that acute nociceptive thresholds increase [13], decrease [2] and do not change [5] with age. Moreover, inconsistent data are also reported concerning the response to tonic painful stimulation and pain inhibitory actions [3].

The present study aims to characterize the influence of chronic stress on nociception with a triple purpose: to evaluate the effect on (i) pain perception (nociceptive threshold) and (ii) tolerance to pain (variation of the nociceptive threshold following a short successive exposition to the same noxious stimulation), and to analyze (iii) the influence of age on both pain perception and tolerance to pain. A model of chronic unpredictable stress was chosen in order to avoid habituation to the stressor and to maintain robust the stress response throughout the experiment [16].

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All regulations determined by the local veterinarian committee (in accordance with the European Community Council Directive 86/609/EEC) concerning the handling of laboratory animals and the international ethical guidelines for the study of experimental pain in conscious animals [19] were followed. Adult male Wistar rats (Charles Rivers, Barcelona, Spain) were assigned to one of the following four groups: (i) young non-stressed controls (YNS) ($n = 8$; 3 months old) and (ii) old non-stressed controls (ONS) ($n = 8$; 18 months old) – both groups were maintained under standard laboratory conditions; (iii) young chronic stressed animals (YCS) ($n = 8$; 3 months old) and (iv) old chronic stressed animals (OCS) ($n = 8$; 18 months old) – both groups were submitted during a period of 21 days to a chronic unpredictable stress paradigm that consisted of applying one of several stressors (one stressor per day) in a random order: forced swimming (three times in a 3 min bath with a 2 min rest between each; water at ±20 °C), overcrowding (1 h), restraint (30 min) and placement on a vibrating/rocking platform (1 h). As shown previously [16], this stress inducing regimen results in chronically elevated serum corticosterone titers throughout the experimental period.

Alteration in pain perception was studied using the tail-flick (TF) test (Model 7360, Ugo Basile, Comerio, Italy), which is used to assess reflexive nociceptive thresholds. Rats were tested on day 21 at 11:00 a.m. by being submitted to three TF tests within a 2 min interval in order to test for tolerance to pain stimulation. To avoid bias related to the handling and testing of the rats, a 1 week period prior to the first nociceptive test was established for the habituation of the animals to the behavioral test equipment. Animals were placed daily in the test room for 2 h followed by a 1 min handling and 1 min training session in the TF apparatus (without performing the test).

The statistical evaluation of the results was made as follows: (a) the nociceptive behavior in old/young rats in stressed/non-stressed groups was analyzed by performing a two-way ANOVA (considering age and treatment as independent variables); (b) the variations of TF latencies trial by trial (from the first to the third trial) were analyzed by Student's *t*-test on young and old groups of animals. Differences between means were considered statistically significant when $P < 0.05$.

Chronic stress induced significant increases in TF latencies at two levels: (a) between the mean TF latency of non-stressed and stressed animals; and (b) between the first and third TF tests of stressed animals. In fact, TF latency increased significantly between YNS and YCS animals (Fig. 1) and between ONS and OCS animals [two-way ANOVA, $F_{(1)} = 14,567$, $P < 0.001$] (Fig. 1). Importantly, the analysis of TF latencies trial by trial also revealed a significant increase in the nociceptive threshold from the first to the third trial in both young and old stressed animals (*t*-tests, $P = 0.016$ and $P = 0.027$, respectively) but not in the control groups (*t*-tests, $P = 0.99$ and $P = 0.93$, respectively) (Fig. 2). The ANOVA analysis failed to reveal any effect of age on nociceptive threshold [$F_{(1)} = 1,355$, $P = 0.24$].

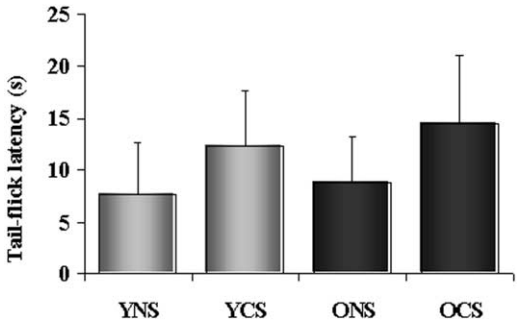

Fig. 1. Effect of chronic unpredictable stress on the nociceptive behavior of YNS and YCS rats and of ONS and OCS animals. Mean TF latency on day 21 of treatment. Mean ± SD.

Overall, this study shows that chronic unpredictable stress inhibits pain-like behavior by increasing the nociceptive threshold. Moreover, the strong analgesia observed from the first to the third noxious stimulation presented in a very short period of time, in animals never tested before, illustrates a remarkable tolerance to a painful stimulus induced by this model of chronic stress. Finally, this helplessness-like effect of chronic stress on pain tolerance was demonstrated in both young and old animals.

Individuals exposed to stressful conditions show an increased nociceptive threshold, known as stress-induced analgesia, which may involve a different neurochemical basis related to stress severity [18]. Consistent with these findings, we have observed that stressed rats (either young

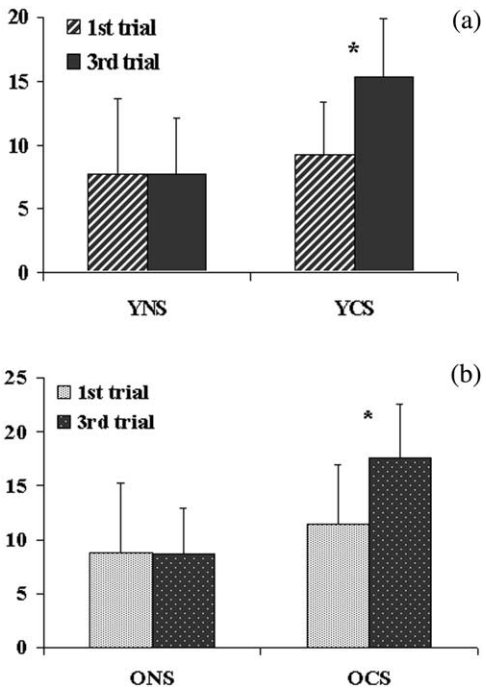

Fig. 2. Mean TF latency on day 21 of the first and third successive TF tests applied to young (a) and old (b) animals. Mean ± SD. *$P < 0.05$.

or old) show higher TF latencies when compared to unstressed controls. Although the influence of stress upon pain perception has been extensively reproduced in several laboratories using different acute stress paradigms [18], the same tests in chronic stressed animals produced contradictory results: some studies report decreased pain perception in chronically stressed subjects [1], while others demonstrate the opposite effect (hyperalgesia) [4]. This apparent paradox might, at least partially, result from differences in the experimental procedures: indeed, whilst studies reporting stress-induced analgesia presented repeatedly the same stressor, others (like the one herein used) presented multiple stressors in an unpredictable paradigm. One likely consequence of these two different protocols (single vs. multiple stressors) is a remarkable difference in the development of habituation to stress [4,14]; indeed, repeated exposure to the same aversive stimulus leads to an attenuated stress response in these subjects [10], while this response is still robust in those facing unpredictable paradigms [16].

Besides the maintenance of robust stress responses during the entire experimental process, chronically stressed animals displayed increased tolerance to pain, that is, a decreased nociceptive reaction to painful stimulation. Indeed, the re-exposure twice to a TF test in the 2 min after the first exposure produced a remarkable increase in latency in chronically stressed animals; although age-related differences in pain responses have been shown with conflicting results [3], this effect was not dependent on the age of the subjects, as it was observed both in young and old animals. Taking into consideration the short period mediating the three tests (2 min), it becomes evident that the mechanisms responsible for the phenomena cannot be directly ascribed to corticosteroid receptor activation (which involves activation of nuclear receptors). Alternatively, the sustained hypercorticalism is likely to alter other neurotransmitter systems/levels. Amongst these opioids are a likely candidate, as was shown in a potentiation of morphine analgesia (TF and hot-plate tests) in rats given inescapable shock prior to pain measurements but not in animals exposed to escapable shocks or restraint stress [17]. These findings confirm that uncontrollable stress enhances morphine analgesia [7]. Thus, if inescapable shock leads to opioid release and subsequent analgesia, a similar mechanism might occur in our stressed animals: more specifically, the first exposure to the TF test may trigger an abnormally high release of opiates within the CNS in stressed animals, and the re-exposure to the test in the next 2 min would still occur under an analgesic state, thereby accounting for the increased tolerance to painful stimuli. However, experiments involving the administration of opioid antagonists before the exposure to successive noxious stimulation are needed in order to test this hypothesis.

Evidence has recently shown that pain perception is the result of a fine balance between descending antinociceptive (inhibiting) and pronociceptive (facilitating) actions upon spinal nociceptive transmission. In fact, manipulations of different supraspinal pain control centers have revealed the existence of nuclei that inhibit or increase [9,12] pain behavior. Disruption of the neurotransmitter balance regulating the large network of connections between the different components of the supraspinal pain control system may lead to changes in pain perception in the model of chronic unpredictable stress used in this study.

Furthermore, it was proposed that morphological rearrangements in the brain structures (e.g. hippocampus) brought on by various types of allostatic load might influence the cognitive appraisal of the pain response [11]. While previous studies showed a stress-induced structural reorganization of the hippocampal formation, the present study confirms that pain threshold is altered in chronically stressed subjects. These findings suggest that as a consequence of indirect long-term activation of the HPA axis by stressful stimuli, central mechanisms of pain perception are likely to be impaired. Ultimately, a more detailed evaluation of the morphofunctional consequences of chronic stress and pain in the limbic system might improve the knowledge of the motivational-affective component of pain and help in the design of better therapeutic strategies for the many subjects currently suffering from chronic pain.

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Chapter 2.1.2

Pinto-Ribeiro F, Moreira V, Pêgo JM, Leão P, Almeida A, Sousa N.

Antinociception induced by chronic glucocorticoid treatment is correlated to local modulation of spinal neurotransmitter content.

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Antinociception induced by chronic glucocorticoid treatment is correlated to local modulation of spinal neurotransmitter content

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Abstract

Background: While acute effects of stress on pain are well described, those produced by chronic stress are still a matter of dispute. Previously we demonstrated that chronic unpredictable stress results in antinociception in the tail-flick test, an effect that is mediated by increased levels of corticosteroids. In the present study, we evaluated nociception in rats after chronic treatment with corticosterone (CORT) and dexamethasone (DEX) in order to discriminate the role of each type of corticosteroid receptors in antinociception.

Results: Both experimental groups exhibited a pronounced antinociceptive effect after three weeks of treatment when compared to controls (CONT); however, at four weeks the pain threshold in CORT-treated animals returned to basal levels whereas in DEX-treated rats antinociception was maintained. In order to assess if these differences are associated with altered expression of neuropeptides involved in nociceptive transmission we evaluated the density of substance P (SP), calcitonin gene-related peptide (CGRP), somatostatin (SS) and $_{B2}$-γ-aminobutiric acid receptors ($GABA_{B2}$) expression in the spinal dorsal horn using light density measurements and stereological techniques. After three weeks of treatment the expression of CGRP in the superficial dorsal horn was significantly decreased in both CORT and DEX groups, while $GABA_{B2}$ was significantly increased; the levels of SP for both experimental groups remained unchanged at this point. At 4 weeks, CGRP and SP are reduced in DEX-treated animals and $GABA_{B2}$ unchanged, but all changes were restored to CONT levels in CORT-treated animals. The expression of SS remained unaltered throughout the experimental period.

Conclusion: These data indicate that corticosteroids modulate nociception since chronic corticosteroid treatment alters the expression of neuropeptides involved in nociceptive transmission at the spinal cord level. As previously observed in some supraspinal areas, the exclusive GR activation resulted in more profound and sustained behavioural and neurochemical changes, than the one observed with a mixed ligand of corticosteroid receptors. These results might be of relevance for the pharmacological management of certain types of chronic pain, in which corticosteroids are used as adjuvant analgesics.

Background

Nociception can be modulated at different levels of the CNS through facilitating (pronociceptive) or inhibiting (antinociceptive) central actions [1-3]. One of the levels where nociceptive modulation takes place is in laminae I–II of the spinal dorsal horn [4], where nociceptors synapse upon interneurons and projection neurons [5,6]. The transmission of nociceptive information in the dorsal horn involves several events, neuropeptides and fibres. After peripheral noxious stimulation of unmyelinated nociceptors the release of calcitonin gene-related peptide (CGRP) [7], substance P (SP) [8] and somatostatin (SS) [4,9] is increased although it remains largely unchanged after innocuous stimulation or stimulation of large myelinated fibres [8,9]. Spinal nociceptive neurons that are excited by CGRP and SP [10,11] receive numerous synaptic contacts from primary afferent terminals colocalizing these neurotransmitters, whereas non-nociceptive neurons lack synaptic input from boutons with both peptides [12]. Spinal SS [13] and GABA [14] have an inhibitory effect on nociceptive neurons, being present mainly in fibres belonging to local inhibitory interneurons [15].

Acute stress induces analgesia but the effects of chronic stress in nociception are still controversial, with studies reporting hyperalgesia after prolonged stress [16], while others observed analgesia [17]. Recently, we demonstrated that animals submitted to chronic unpredictable stress display antinociception in the tail-flick test [18]; since the plasmatic levels of corticosteroids were increased throughout the entire experimental period, we implicated these hormones in that phenomenon. Corticosteroids can bind to two types of corticosteroid receptors, mineralocorticoid (MR) and glucocorticoid (GR) receptors. In basal conditions, MR display greater occupancy than GR; thus, conditions resulting in elevation of corticosteroids, e.g. stress, will result mainly in increased activation of GR. Importantly, the spinal cord is a corticoid-responsive tissue [19] and within the spinal cord the greatest density of GR and MR occurs in laminae I–II [20]. Of notice, CGRP and SP (but not SS) coexist with corticosteroid receptors in neurons of dorsal root ganglia [21] and some studies demonstrate that an imbalanced corticosteroid milieu may affect neuropeptide content in the DRG [22,23]. Importantly, corticosteroids are often used as adjuvant analgesics in the management of several types of pain [24-26]. Taken together, these findings predict a potential influence of corticosteroids in the modulation of spinal nociceptive transmission.

In the premise that a distinctive activation of MR or GR could be responsible for altered levels of neuropeptides involved in spinal nociceptive transmission and, consequently, for diverse pain-like effects we evaluated the density of CGRP, SP, SS and $GABA_{B2}$ innervation in the spinal dorsal horn of animals submitted to prolonged administration of CORT (activating both MR and GR) and DEX (a selective ligand of GR). These data were correlated with pain-like behaviour measured through the tail-flick and hot-plate tests.

Results

Pain-like Behaviour

Evolution within groups during the experimental period

Analysis of TF and HP latency in CONT revealed no significant differences between testing sessions throughout the experimental period ($ANOVA_{rm}$, TF, P = 0.29 and HP, P = 0.60).

Tail-flick test

The chronic subcutaneous administration of CORT and DEX resulted in a significant decrease in pain-like behaviour. Statistical data indicate that both CORT and DEX induced a significant increase in TF latencies on day 21 ($ANOVA_{ow}$, P = 0.002, *pos-hoc* Bonferroni, CORT × CONT, $p < 0.05$; DEX × CONT, $p < 0.01$) (Fig. 1A). However, with the prolongation of the treatment (day 28) only subjects under DEX treatment maintained the significant increase in TF thresholds; in contrast in CORT-treated animals nociceptive behaviour decreased slightly ($ANOVA_{ow}$, P = 0.0003, *pos-hoc* Bonferroni, CORT × CONT, $p > 0.05$, DEX × CONT, $p < 0.001$ and DEX × CORT, $p < 0.001$) (Fig. 1B).

Hot-plate test

The prolonged administration of DEX but not CORT resulted in a significant decrease in nociceptive behaviour. Statistical data indicates that HP latencies are significantly increased in DEX-treated animals on day 28 ($ANOVA_{ow}$, P = 0.02, *pos-hoc* Bonferroni, DEX × CONT, $p < 0.05$) (Fig. 1D). Contrary to what was observed for the TF test, no differences between groups were observed on day 21, although a trend towards an antinociceptive effect was already observed ($ANOVA_{ow}$, P = 0.07) (Fig. 1C).

Neurotransmitter Spinal Innervation

All statistical data presented in this section referring to immunoreactivity evaluation is based on the study of the lumbar portion of the spinal cord as no differences in neurotransmitter-IR were found between cervical and lumbar portions.

Stereology

The stereological analysis of CGRP-, SP-, SS- and $GABAB_2$-IR in the spinal dorsal horn after prolonged CORT and DEX treatment is summarized in figure 2. The expression of CGRP-IR was significantly decreased in both DEX and CORT-treated animals when compared to CONT on day 21 ($ANOVA_{2\,w}$, $p < 0.0001$, pos-hoc Bonferroni, CORT × CONT, $p < 0.01$ and DEX × CONT, $p < 0.001$) (Fig. 2A) although this effect was sustained only in DEX animals on

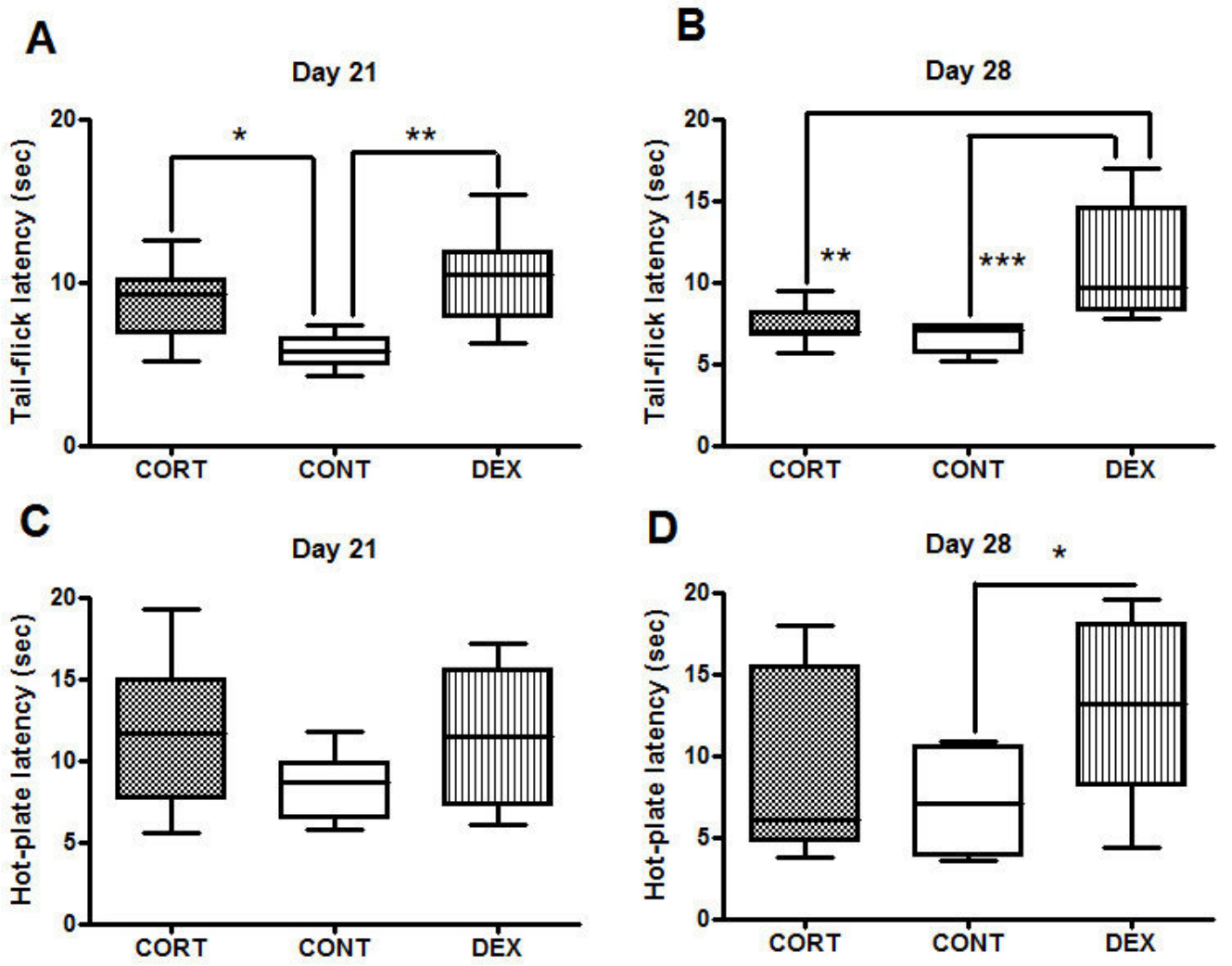

Figure 1

Nociceptive behaviour. Tail (A, B) and paw (C, D) withdrawal latency after chronic corticosteroid treatment for 21 (1) and 28 (2) days with CORT and DEX. Both CORT and DEX groups display higher TF latencies after 21 days of treatment (A, B) although this effect is only sustained by DEX group at the end of the experiment (B); note that only DEX induces an increase in hind-paw latency and only after 28 days of treatment (D). (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

day 28 ($ANOVA_{2\ w}$, $p < 0.036$, pos-hoc Bonferroni, DEX × CONT, $p < 0.05$) (Fig. 2E). The level of SP-IR in CORT and DEX was not significantly different from CONT on day 21 ($ANOVA_{2\ w}$, $P = 0.70$) (Fig. 2B) but in DEX-treated animals there was a significant decrease in SP expression on day 28 ($ANOVA_{2\ w}$, $P = 0.033$, pos-hoc Bonferroni, DEX × CONT, $p < 0.05$) (Fig. 2F). No changes were observed between experimental groups in what concerns SS-IR in the spinal dorsal horn ($ANOVA_{2\ w}$, day 21, $P = 0.86$ and day 28, $P = 0.88$) (Figs. 2C, G). In DEX-treated animals $GABA_{B2}$-IR is significantly increased in both 21 and 28 days ($ANOVA_{2\ w}$, $p < 0.0001$, DEX × CONT, day 21, $p < 0.001$ and day 28, $p < 0.01$). A similar increase was observed in the CORT-group on day 21 ($ANOVA_{2\ w}$, $p < 0.0001$, CORT × CONT, day 21, $p < 0.001$ and day 28, $p > 0.05$) but $GABA_{B2}$ to returned to basal levels on day 28 (Figs. 2D, H).

Densitometric Measurements

The results obtained through the densitometric analysis of CGRP, SP, SS and $GABAB_2$-IR were closely related to those obtained through the stereological quantification both for day 21 (Pearson analysis, $CGRP_{21\ days}$, $r = 0.98$ and $p < 0.0001$; $SP_{21\ days}$, $r = 0.98$ and $p < 0.0001$; $SS_{21\ days}$, $r = 0.99$ and $p < 0.0001$; $GABA_{B2,21\ days}$, $r = 0.97$ and $p < 0.0001$) (Figs. 3A, B, C, D) and day 28 (Pearson analysis, $CGRP_{28\ days}$, $r = 0.94$ and $p < 0.0001$; $SP_{28\ days}$, $r = 0.98$ and $p < 0.0001$; $SS_{28\ days}$, $r = 0.94$ and $p < 0.0001$; $GABA_{B2,28\ days}$, $r = 0.95$ and $p < 0.0001$) (Figs. 3E, F, G, H).

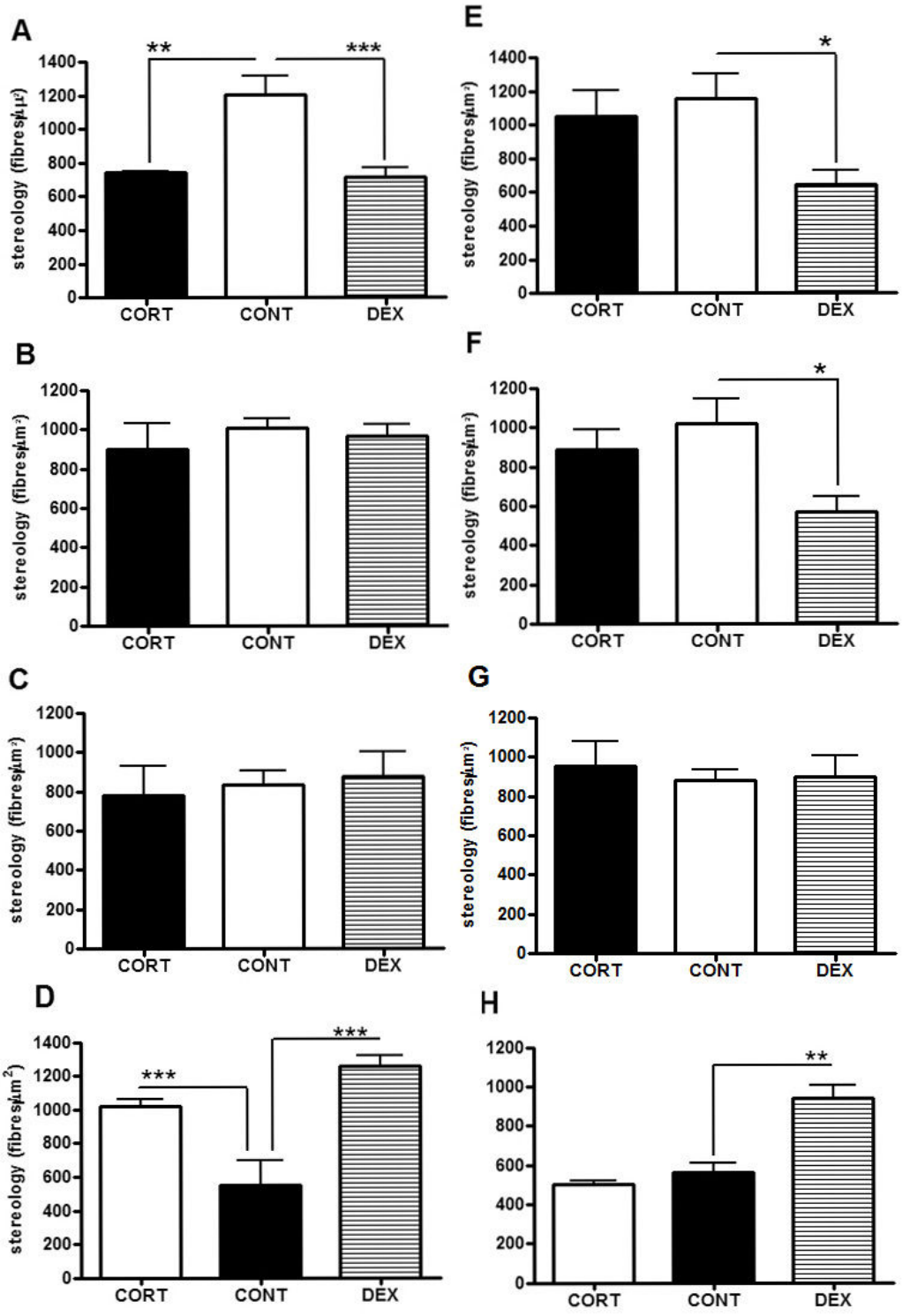

Figure 2
Neuropeptide and receptor expression in the spinal dorsal horn. Immunoreactive content in the dorsal horn of the spinal cord after 21 and 28 days of chronic corticosteroid treatment. (CGRP(A, E), SP(B, F), SS(C, G) and $GABA_{B2}$(D, H); *p < 0.05, **p < 0.01 and ***p < 0.001).

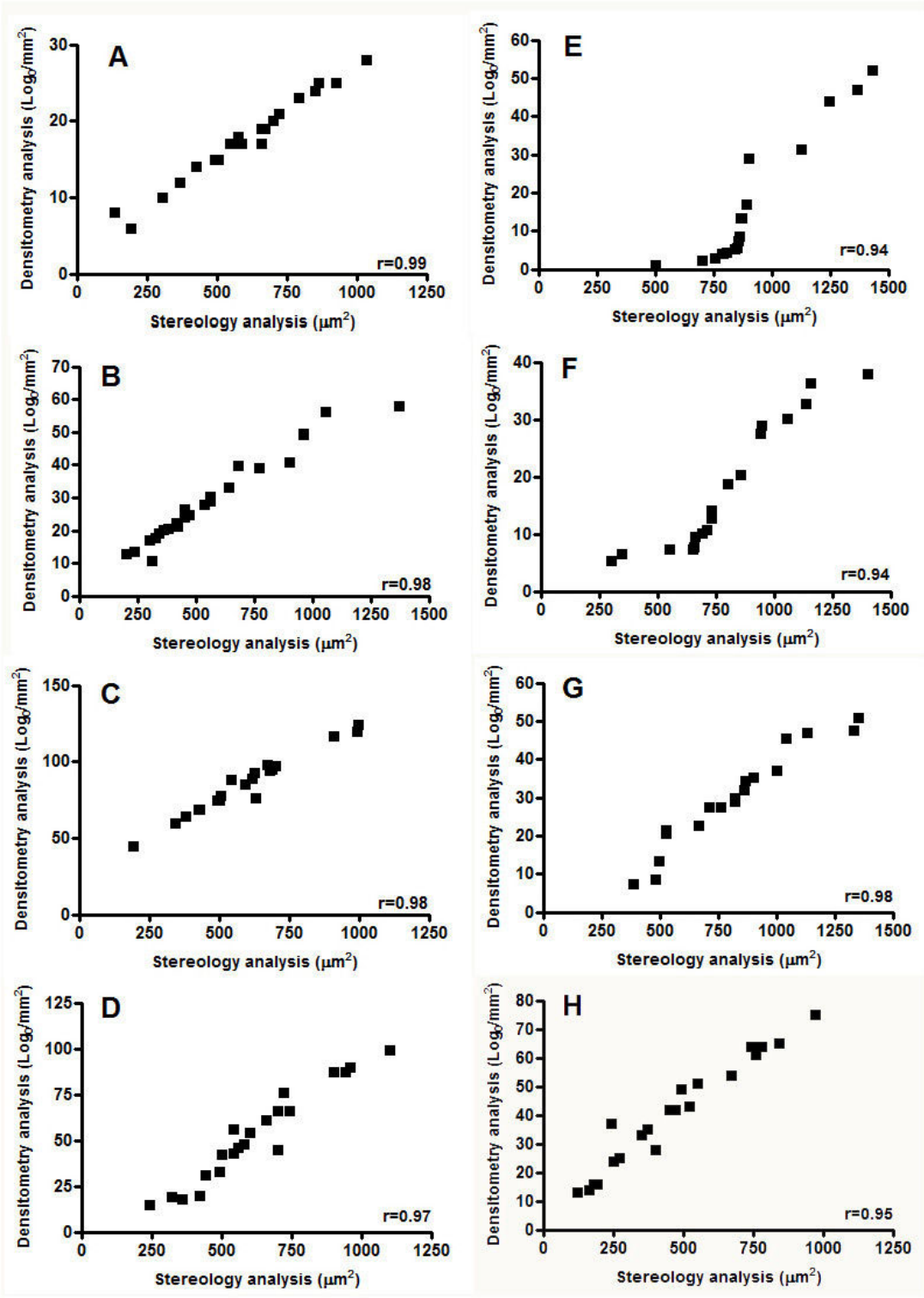

Figure 3

Pearson correlation between densitometry and stereology quantification methods. Pearson correlation for validation of densitometric versus stereologic quantification methods on days 21(A-D) and 28(E-H). (SS(A, E), SP(B, F), CGRP(C, G) and $GABA_{B2}$(D, H)).

Discussion

The present study demonstrates that prolonged administration of corticosteroids decreases nociception. The antinociceptive effect reflects both a decrease of pronociceptive neuropeptide expression and an increased availability of GABA receptors in laminae I–II of the spinal dorsal horn. After 21 days of treatment the decrease in pain-like behaviour was correlated with a decrease in CGRP and an increase in $GABA_{B2}$ receptors in the spinal cord of CORT and DEX treated-animals. Interestingly, the antinociceptive effect in the CORT-group vanished after four weeks of treatment (which was paralleled by a restoration of CGRP and $GABA_{B2}$ expression towards control levels) while it remained unchanged in DEX-treated rats (which were correlated with a decrease in spinal content of both CGRP and SP and increased availability of $GABA_{B2}$). These findings confirm that corticosteroid receptors play a crucial role in the mediation of pain transmission at the spinal cord level.

Pain perception involves the transmission of nociceptive messages from the periphery to the CNS. This transmission can be modulated by acute [16] and chronic stress [16,17]. Recently, we showed that chronic unpredictable stress, which results in a prolonged elevation of plasmatic glucocorticoid (GC) levels, decreases pain-like behaviour [18]. Most actions mediated by chronic stress are attributed to hypercortisolemia, as the increased secretion of corticosteroids characterizes the sustained phase of the stress response [27]. Because corticosteroids can bind to two types of receptors we decided to further explore the role of each of these corticosteroid receptors on the nociceptive modulation. It is important to note at this point, that the confounding effect of drug potency has been considered, as the doses of each corticosteroid were adjusted accordingly to their glucocorticoid potency. Thus, in this experimental paradigm CORT treatment differs from DEX administration basically in terms of MR activation: while CORT treatment activates these receptors, DEX does not bind to MR and because it shuts-off the endogenous secretion of corticosteroids, MR remains unoccupied [28].

The results observed after prolonged daily treatment with corticosteroids demonstrate that these steroids promote antinociception. GR are likely to mediate this phenomenon since a similar response was observed in DEX-treated animals. The TF test evaluates a spinally organized reflex [29,30] mediated by C-fibres innervating the tail [31,32] and motoneurons innervating the three sets of back muscles that control tail movements [33-35]. In contrast, the HP test involves a supraspinally integrated response, and thus, represents a more complex behavioural response [36]. Such difference in the neuroanatomical substrates implicated in both tests might explain why there was only a trend towards increased HP latencies after 21 days of CORT and DEX treatments.

It is admissible that the influence of GC upon neuropeptidergic innervation results both from direct and indirect actions. Indirect actions may result from altered availability of $GABA_{B2}$ receptors in CORT and DEX treated animals on day 21, as $GABA_B$ receptors are well known players in pain modulation [15,37]. Moreover Kangrga and collegues [38] described that the antinociceptive effect of GABAergic transmission in the spinal dorsal horn results from presynaptic inhibition of the release of excitatory amino acids and neurotransmitters from the primary afferents [14,39] which is in accordance with our observations that CGRP, a pronociceptive neuropeptide, is decreased in both CORT- and DEX-treated groups. An alternative indirect action of glucocorticoids might occur through the modulatory actions of arachidonic pathways which down-regulate nerve growth factor (NGF); this, in turn, is known to exert an inhibitory effect in both the accumulation and release of CGRP mRNA in nociceptors [40,41]. In parallel, the direct modulation of glucocorticoids can be ascribed to the fact that approximately one third of the afferents that are immunoreactive to SP or CGRP, also display immunoreactivity to GR [21]. Thus, it is plausible to assume that GR activation of nuclear responsive elements alters the expression of such transmitters in spinal dorsal horn afferents. This hypothesis is further supported by the fact that it was recently shown that stressors decrease CGRP expression in the frontal cortex, hippocampus, occipital cortex and hypothalamus [42].

Curiously, the dissimilarity in pain-like behaviour observed between CORT and DEX groups after 28 days of treatment, suggests that other mechanisms involving MR activation are implicated in the modulation of pain. In fact, the behavioural differences observed between CORT and DEX treatment at 28 days were paralleled by distinct patterns in CGRP, SP and $GABA_{B2}$ expression in the superficial dorsal horn: while in CORT-treated animals the expression of both CGRP and $GABA_{B2}$ was restored to control levels, DEX treatment resulted in a decreased expression of SP and CGRP and sustained increase in $GABA_{B2}$. The explanations for such discrepancy are more complex, as besides the local effects at the spinal cord level, they might involve alterations at the supraspinal level. Indeed, there is a complex feedback system between the neurotransmitters herein studied and GC involving supraspinal processing that is regulated by MR. There is evidence that the activation of MR is correlated with GABA modulation [43] in lamina II [44] of the spinal cord, namely in interneurones [45], and in other supraspinal pain modulating areas such as the rostroventral lateral medulla (RVM) [46] or the periaductal grey matter (PAG) [47].

This effect of GABAergic transmission appears to selectively inhibit the release of SP, but not of CGRP, [15] which may account at least partly for the differences observed between the groups. Another alternative, but not exclusive, mechanism to explain the differential effect of DEX and CORT upon neuropeptidergic spinal expression derives from the specific modulatory effects of MR upon preprotachykinin (PPT), the precursor of SP expression; in fact, MR activation has been shown to positively regulate (up to 50%) mRNA PPT expression in the nervous tissues [48]. The more persistent changes in pain perception induced by DEX treatment and measured by an increase in both the TF and HP latency at day 28 might therefore result from a decrease in SP fibre innervation in the spinal dorsal horn.

Contrary to CGRP, SP and $GABA_{B2}$, no effect of GC was observed on the spinal levels of SS. This differential change observed between these neuropeptides illustrates the selectivity of this process, and is likely to be related with the lack of coexistence of corticosteroid and SS in the spinal cord [21]. Interestingly, different neurotransmitters are associated with different roles in pain modulation [5,49]. In contrast to CGRP/SP, SS is a tonic inhibitor of peripheral nociceptors [50]. Thus, the data herein reported suggests that the effects of chronic corticosteroid treatment on pain perception are associated with changes in the nociceptive transmitting system (CGRP/SP) but would not involve specific alterations in the spinal intrinsic modulatory system (SS).

In addition to their presence in the spinal dorsal horn, both glucocorticoid- [51] and mineralocorticoid- [52] receptors are present also in neurons of a large number of supraspinal sites along the rostrocaudal extent of the neuraxis in the rat. These include several forebrain and brainstem components of the supraspinal pain control system, including areas like the anterior cingulate cortex [53], amygdala [54], paraventricular hypothalamic nucleus [55], periaqueductal grey matter [56], locus coeruleus [57], rostral ventromedial medulla [58], dorsal reticular nucleus [59] and caudal ventrolateral medulla [60]. Taking into account data obtained in the present study on the effect of corticosteroid manipulation upon spinal neurotransmitter content, future studies should explore alterations induced at supraspinal levels. Accordingly, profound structural, physiological and neurochemical alterations have been observed at different forebrain areas following chronic manipulation of corticosteroids [30,61-63].

Conclusion

The present study shows that corticosteroids modulate nociception by altering the expression of neuropeptides involved in nociceptive transmission at the spinal cord level. Moreover, we demonstrate differential modulatory actions of different ligands of corticosteroid receptors, which are of relevance for the pharmacological management of those conditions involving chronic pain, in which corticosteroids are recommended as adjuvant analgesics.

Methods

Subjects

Wistar Han rats obtained from Charles Rivers (Barcelona, UE), weighting between 200–240 g, at the beginning of the experiment, were housed in groups of three in standard polycarbonate cages (45.4 × 25.5 × 20 cm). The light cycle was 12:12 h with lights on at 9:00 am and housing was maintained at 22°C and 30% relative humidity. Water and food were available ad libitum. All regulations determined by the local veterinarian committee (in accordance to the European Community Council Directive 86/609/EEC) concerning the handling of laboratory animals and the international ethical guidelines for the study of experimental pain in conscious animals were followed [64].

Chronic corticosteroid treatment

Corticosterone, dexamethasone and sesame oil were acquired from Sigma (St Louis, MO, USA). Subjects were assigned to one of the following three groups (n = 24):

(i) Controls (CONT). Rats were submitted to vehicle injection (0.5 ml sesame oil) everyday (05:00 pm), during 3 weeks (n = 4) and 4 weeks (n = 4).

(ii) Corticosterone-treated (CORT). Rats were submitted over a period of 3 weeks (n = 4) and 4 weeks (n = 4) to a daily subcutaneous injection (05:00 pm) of 40 mg/kg dose of 4-Pregnene-11β,21 diol-3,20-dione in sesame oil.

(iii) Dexamethasone-treated (DEX). Rats were submitted over a period of 3 weeks (n = 4) and 4 weeks (n = 4) to a daily subcutaneous injection (05:00 pm) of 300 μmg/kg dose of 9α-fluoro-16α-methylpredenisolone in sesame oil.

Nociceptive testing

Pain-like behaviour was analyzed for each animal using the tail-flick (TF) and the hot-plate (HP) tests. In the TF (Ugo Basile, Comerio, Italy) the time spent the start of the stimulus and the withdrawal of the tail (nociceptive latency) was recorded, whereas in the HP (Ugo Basile, Comerio, Italy) as the heating plate was kept at a constant temperature of 54 ± 0.5°C, it was the latency for hind paw licking or jumping was recorded.

In order to determine the nociceptive threshold, rats were tested before corticosteroid administration (day 0) and on

days 7, 14, 21 and 28 of the treatment; each testing day animals were submitted (11:00 am) to 3 TF tests, within a 2 min interval, and 2 HP test, with 45 min interval (Fig. 4). To avoid bias related with the handling and testing of the rats, a one-week period prior to the first nociceptive test was established for the habituation of the animals to the behavioural test equipment and the researcher. Animals were placed daily in the test room for 2 h followed by a 10 minute handling and 1 minute training session in the TF and the HP apparatus (without performing the test).

Immunocytochemistry

At the end of the experimental period (21 or 28 days), animals were anesthetized intraperitoneally (sodium pentobarbital, 0.5 mg/kg) and perfused transcardially with 4% paraformaldehyde in PBS 0.1 M, pH 7.2. The spinal cord was removed and placed in 30% sucrose for 24 h. Portions of the cervical and lumbar spinal cord enlargements were sampled. Sections, 30 μm thick, were cut on a vibrating blade microtome (Leica, Germany) and collected in superfrosted slides. Sections from the same region for all subjects and treatments were exposed to the same solutions. Sections were permeabilized for 10 min in 0.2% Triton X-100 in Tris buffer saline (TBS) and microwaved (20 min) while immersed in citrate buffer (0.1 M). Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS (10 min) and non-specific staining was blocked with 4% bovine serum albumin (BSA) in PBS (30 min). Alternating sections were incubated overnight at room temperature in rabbit primary antibodies against CGRP (1:3000; Chemicon, USA) (Fig. 5), SP (1:3000; Chemicon, USA), SS (1:3000; Chemicon, USA) and $GABA_{B2}$ (1:1000, Chemicon, USA) (Fig. 6) in 0.02% Triton X-100 (PBST). Antigen visualization was carried out using a universal detection system (BioGenex, San Ramon, CA) and diaminobenzidine (DAB; 0.025% and 0.5% H_2O_2 in Tris-HCl 0.05 M, pH 7.2).

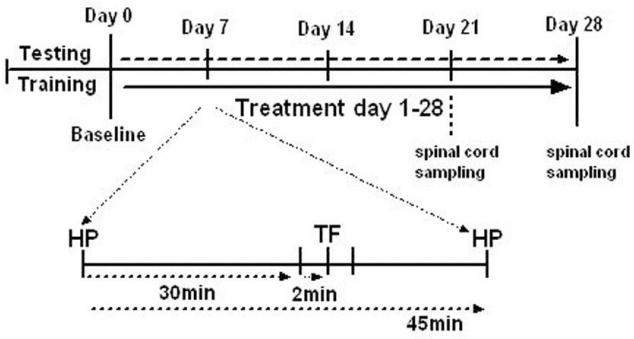

Figure 4
Time course of testing and sampling sessions throughout the four week experimental period.
Within a testing session, tail-flick and hot-plate tests were performed according to the time course example for day 7.

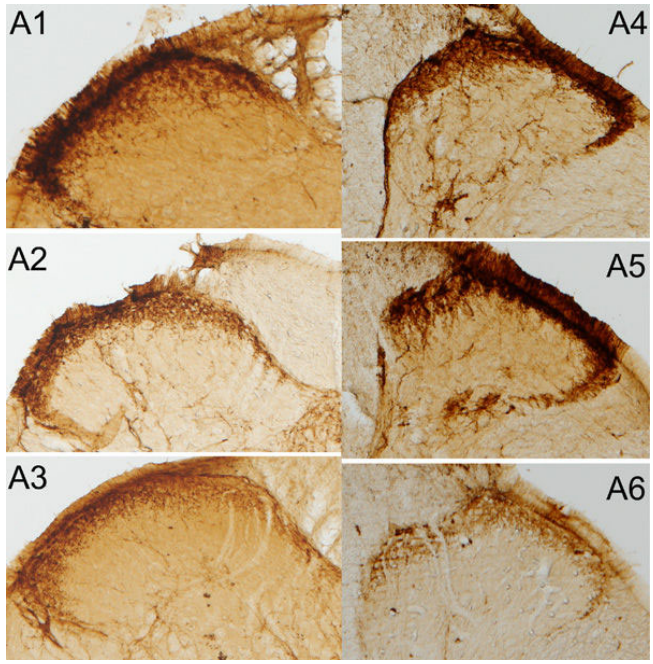

Figure 5
Photomicrographs of superficial dorsal horn sections.
Examples of photomicrographs of superficial dorsal horn sections immunoreacted for CGRP (A) on days 21 (A_{1-3}) and 28 (A_{4-6}) for CONT ($A_{1,4}$), DEX ($A_{2,5}$) and CORT ($A_{3,6}$).

Stereology

The stereological analysis was performed in the dorsal horn of CGRP-, SP-, SS-and $GABA_{B2}$-immunoreacted (IR) spinal cord sections using StereoInvestigator software (MicroBrightField, Williston/VT, USA). From each set of serial sections, ten photomicrographs of areas within the spinal laminae I–II were obtained at a primary magnification of × 50 and analyzed at a final magnification of × 1000. The number of stained fibres per unit of laminae I–II volume (numerical density) was estimated using the optical fractionator method [65]. The surface volume occupied by laminae I–II stained fibres was calculated on the basis of the surface density of the fibres (surface area per unit volume, SV) and the volume of laminae I–II. The SV was estimated, using a 'staggered' cycloid test system in order to obtain the total number of intersections between cycloid arcs and stained fibres. Measurements were made on laminae I–II regions randomly selected by the software.

Densitometric Measurements

The densitometric analysis was performed in the dorsal horn of CGRP, SP, SS and $GABA_{B2}$-IR spinal cord sections using a Zeiss light microscope coupled to a PC, using NIH

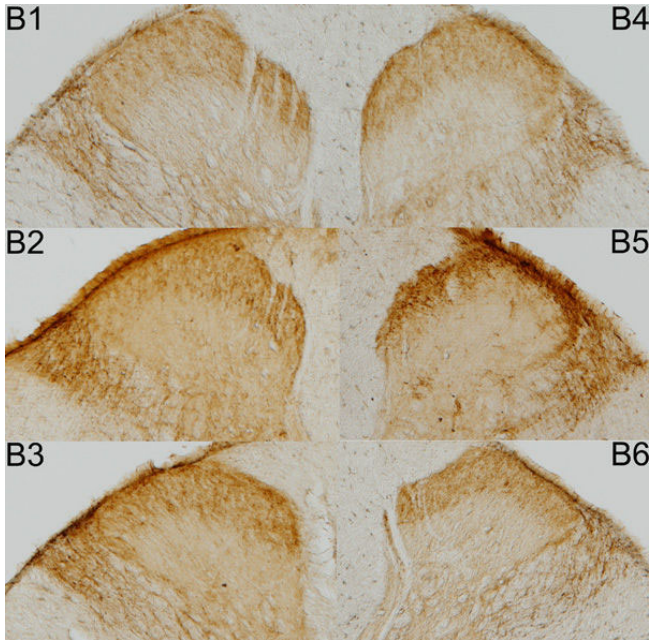

Figure 6
Photomicrographs of superficial dorsal horn sections. Examples of photomicrographs of superficial dorsal horn sections immunoreacted for $GABA_{B2}$ (B) on days 21 (B_{1-3}) and 28 (B_{4-6}) for CONT ($B_{1,4}$), DEX ($B_{2,5}$) and CORT ($B_{3,6}$).

Image 1.52 software. The sampling area for optical density measurement corresponded to all the area occupied by laminas I and II of the spinal dorsal horn, bilaterally. Density levels and distribution of CGRP-, SP-, SS- and $GABA_{B2}$-IR were quantified and, for all sections, background density measurements were subtracted to these values.

Data analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA). A two-way ANOVA ($ANOVA_{2\ w}$) was used to analyze differences between groups at different time points, while repeated-measures ANOVA ($ANOVA_{rm}$) was used to evaluate efficiency of treatment along different time points within groups; pos-hoc Bonferroni's test was used to detect significant differences for both ANOVA analysis. Densitometric and stereological data was compared using the Pearson correlation analysis. Differences were considered statistically significant when $p < 0.05$. All values are presented as mean ± SD.

Abbreviations

CGRP: Calcitonin gene-related peptide; CONT: Controls; CORT: Corticosterone; DEX: Dexamethasone; $GABA_{B2}$: B2-γ-aminobutiric acid receptors; GC: Glucocorticoid; GR: Glucocorticoid receptor; HP: Hot-plate test; IR: Immunoreactivity; MR: Mineralocorticoid receptor; SP: Substance P; SS: Somatostatin; TF: Tail-flick test

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FPR performed the statistics analysis, carried out the IHC and quantification of densitometry and drafted the paper. VM: performed behavioural tests and the stereological analysis. JMP: performed corticosteroid administration and behavioural testing. PL: performed corticosteroid administration and stereological quantification. AA: conceived, designed and coordinated the study and revised the paper. NS: conceived, designed and coordinated the study and revised the paper. All authors read and approved the final manuscript.

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Chapter 2.1.3

Pinto-Ribeiro F, Amorim D., Monteiro AM, Sousa N, Pertovaara A, Almeida A

Chronic unpredictable stress alters the activity of nociceptive modulatory neurones in the rostral ventromedial medulla.

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ABSTRACT

Descending pain modulation is strongly associated with the activity of presumptive pronociceptive ON-cells and antinociceptive OFF-cells of the rostral ventromedial medulla (RVM) (1). We have previously demonstrated that chronic unpredictable stress (CUS) is antinociceptive as it decreases tail-flick latency (2), an effect that was correlated to changes in the neurochemistry of the spinal cord (3). In order to evaluate possible plastic changes in supraspinal pain control centres caused by CUS, we recorded the spontaneous and noxious-evoked activity of ON- and OFF-cells in the RVM. CUS-treated animals displayed increased tail-flick latencies from the third week onwards when compared to control (CTRL) animals. A first analysis of the number of active neurones in the RVM revealed a significant decrease in the relative number of ON-and OFF-cells and an increase in NEUTRAL-cells in the RVM of CUS-treated animals when compared to CTRL. A significant decrease in the number of these cells could alter the ability of this area to modulate nociception and partly contribute to the analgesic effect of stress. In what concerns the response to peripheral stimulation, neurones in the RVM were classified as wide-range-dynamic (WDR – responsive to innocuous and noxious stimuli) and nociceptive specific (NS – responsive only to noxious stimuli) receptive neurones. Our study focused on the latter as no change in either the number of cells or in their spontaneous activity was observed for RVM WDR-responsive ON-, NEUTRAL- and OFF-cells. Overall, the activity of RVM NS responsive neurones was impaired by CUS as a significant decrease in their spontaneous activity was observed. In what concerns noxious-evoked activity, the decreased activity of pronociceptive RVM ON-cells, but not OFF-cells, in CUS-treated animals during thermal noxious stimulation, is in accordance with the stress-induced analgesia observed in behavioural experiments.

These results indicate that CUS induces profound plastic changes in the RVM by both altering the number of nociceptive-responsive cells in the RVM and their spontaneous and noxious-evoked activity. Future studies should evaluate if the antinociceptive effect of CUS is mediated by the main effector of the stress-response, the paraventricular nucleus of the hypothalamus, which is known to have an antinociceptive role (4).

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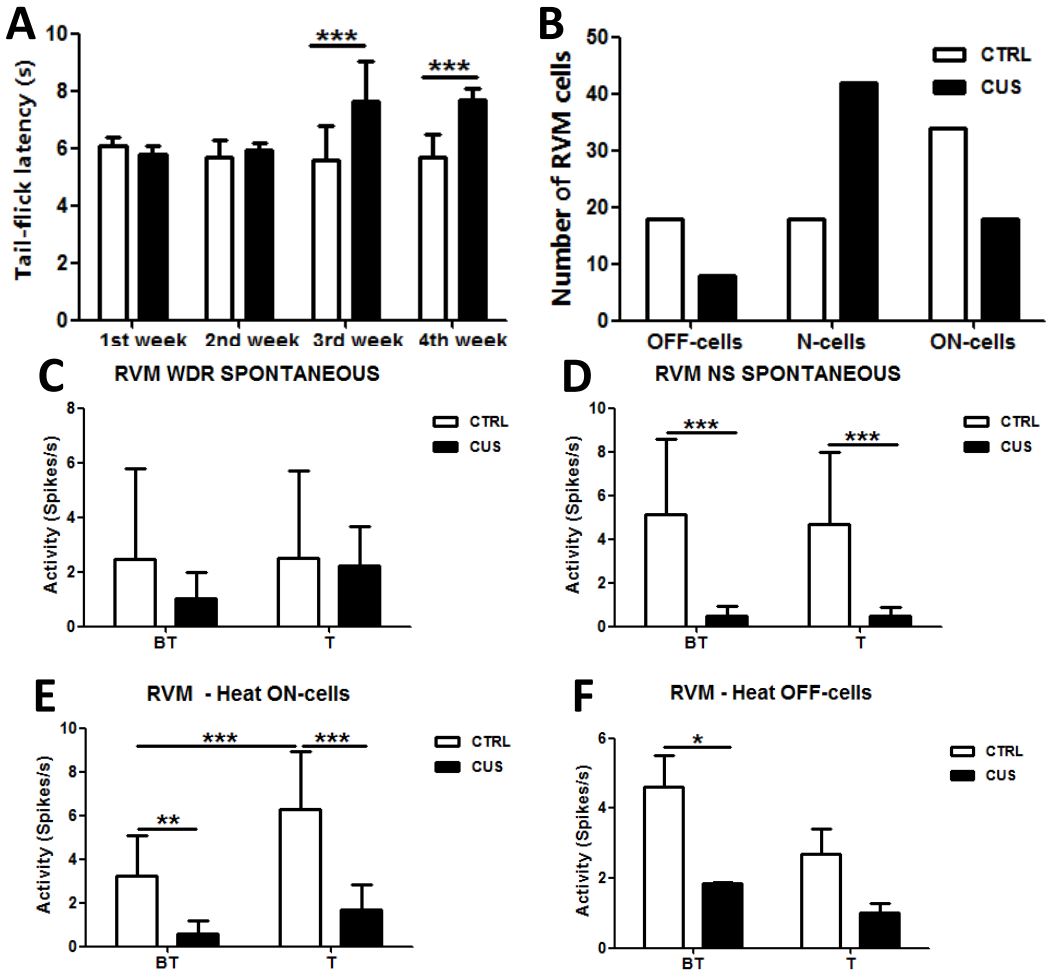

Figure 1 – Behavioural and RVM-electrophysiological evaluation of CTRL and CUS-treated rats during noxious stimulation. Tail-flick latency of CUS-treated animals increases in comparison to CTRL from the third week onwards (A). The number of active RVM ON- and OFF-cells is significantly decreased in CUS-treated animals when compared to CTRL, while the opposite occurs for NEUTRAL-cells (B) (CHI-test, $p<0.0001$). In the analysis of the neuronal activity of RVM neurones before (BT) and during noxious peripheral stimulation (T) in CTRL and CUS animals, neurones were first divided in wide-range dynamic (WDR) (C) and nociceptive-specific (NS) (D) receptive neurones. Overall, CUS decreased the activity of NS ($ANOVA_{2W}$, $F_{(1,102)}=100.8$, $p<0.0001$) (D) but not WDR neurones (C) ($ANOVA_{2W}$, $F_{(1,63)}=2.303$, $p=0.13$). Additionally, CUS decreased the spontaneous activity of ON- ($ANOVA_{2W}$, $F_{(1,44)}=13.15$, $p<0.01$; BT, $p<0.001$) and OFF-cells ($ANOVA_{2W}$, $F_{(1,18)}=8.093$, $p=0.011$) (E-F) as well as the heat noxious-evoked activity of ON- (E) (but not of OFF-) (F) cells in the RVM ($ANOVA_{2W}$, $F_{(1,44)}=13.15$, $p<0.001$; T, $p<0.001$). *$p<0.05$, **$p<0.01$ and ***$p<0.001$

Chapter 2.2

Anatomical connections of the PVN with supraspinal pain control centres

Chapter 2.2.1

Pinto-Ribeiro F, Almeida A

Brain connections of the paraventricular nucleus of the hypothalamus (PVN): an anterograde and retrograde tracing study in the rat.

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Brain connections of the paraventricular nucleus of the hypothalamus (PVN): an anterograde and retrograde tracing study in the rat

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Keywords: paraventricular nucleus of the hypothalamus, Cholera Toxin biotynilated, biotynilated-dextran amine, brain connections

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Abstract

The paraventricular nucleus of the hypothalamus (PVN) is involved in the neuroendocrine response to stress, including the activation of the endogenous pain inhibitory systems in stressful conditions, a phenomenon known as stress-induced analgesia (SIA). Despite extensive evidence suggesting the involvement of the PVN in SIA, anatomical data on the neuronal connections of the PVN with the supraspinal pain control system is scattered. The purpose of this study was to make a detailed identification of areas within the brain that are connected to the PVN, with a special emphasis on those that might contribute to its role in pain modulation. Projections to and from the PVN were identified after the iontophoretic microinjection of retrograde (CTb) and anterograde (BDA) anatomical tracers, respectively, in this nucleus.

Stained nuclei and fibres, respectively, were mainly located ipsilaterally in the telecenphalon, diencephalon and mesencephalon. In the forebrain, the bed nucleus of stria terminalis (BDST), lateral septal nucleus, several hypothalamic nuclei (the arcuate, dorsomedial, ventromedial nuclei, and posterior hypothalamic area) and medial preoptic area were the areas presenting the highest number of labelled neurons, followed by periaqueductal gray matter (PAG), deep mesencephalic and gigantocellular nuclei in the brainstem. A large number of stained fibres were found in the agranular insular cingulated and ventral orbital cortices, amygdala, BDST, caudate-putamen, dorsomedial hypothalamic nucleus, anterior and posterior hypothalamic areas, preoptic nuclei, followed by smaller amounts of fibres in the PAG.

These anatomical results are discussed in relation to the neuronal networks involving the PVN in the regulation of different physiological systems.

1. Introduction

The paraventricular nucleus of the hypothalamus (PVN), located in the medial part of the hypothalamus (Paxinos & Watson, 2005) is an heterogeneous area (Swanson and Kuypers, 1980) that integrates inputs from many systems (Ferguson *et al.*, 2008), including the neuroendocrine (Bester *et al.*, 1997), behavioural (Normandin and Murphy, 2008), thermoregulatory (Cham *et al.*, 2006) and cardiorespiratory systems (Swanson and Sawchenco, 1983; Coote, 1987; 1995; Zhong *et al.*, 2008). Additionally, this nucleus mediates the stress response through the activation of the HPA axis (Jankord and Herman, 2008), leading to the release of corticosteroids by the adrenal glands (McEwen & Sapolsky, 1995). While investigating central afferent projections conveying information on acute stress to the PVN, Larsen and Mikkelsen (1995) observed that after a intraperitoneal administration of hypertonic saline, retrogradely *c-fos* activated neurones could be visualized in the subfornical organ, the organum vasculosum laminae terminalis, the ventrolateral division of the medulla oblongata (A1 and C1), the nucleus of the solitary tract (NTS) and the parabrachial nucleus (PBN). In addition, Cullinan and colleagues (1996) verified that after an acute stress stimulus PVN-projecting nuclei included the parastrial, supramammillary and dorsomedial hypothalamic (DMH) nuclei and the medial preoptic, anterior and posterior hypothalamic areas. More recently, studies investigating the circuitry of stress integration demonstrated that areas such as the ventral subiculum, the prefrontal cortex, the medial amygdala, the lateral septum, the paraventricular thalamus and the suprachiasmatic nucleus also project to the PVN (Herman *et al.*, 2002).

Of special interest is the connection between stress and pain perception, as acute stress or life threatening conditions result in profound transient analgesia, known as stress-induced analgesia (SIA), in which the PVN has been partly implicated (Truesdell and Bodnar, 1987; Robinson *et al.*, 2002). From supraspinal pain control centres, the medullary dorsal reticular nucleus (DRt), known to participate in pronociceptive descending control of spinal nociceptive transmission (Lima and Almeida, 2002; Almeida *et al.*, 2006), was shown to project to the PVN (Leite-Almeida *et al.*, 2006). On the other hand, PVN efferent projections terminate in several areas within the pain control system, such as the periaqueductal gray matter (PAG), the raphe magnus (RVM), the NTS (Holstege, 1987; Hardy, 2001), the DRt (Almeida *et al.*, 2002) and the substantia gelatinosa of the spinal cord (Chen & Toney, 2003). Moreover, studies evaluating the pain-activated pathways, through *c-fos* staining (Hunt *et al.*, 1987; Bullit, 1990), also demonstrate that specific

sensory afferents ascend bilaterally from the spinal cord and target the PVN (Akaishi *et al.*, 1988; Palkovitz *et al.*, 1999) amongst other areas. Finally, Bonaz and colleagues (2000) showed that lamina I, V, VII and X nociceptive neurones project directly to the PVN. Finally, PVN neurons activated by noxious stimulation were shown to project directly to the spinal dorsal horn (Condés-Lara *et al*, 2009). According to the anatomic, endocrine and electrophysiological evidence, the PVN was shown to exert an antinociceptive role (Yirmiya *et al.*, 1990) directly upon the spinal dorsal horn (Condes-Lara *et al.*, 2007; 2008; Pinto-Ribeiro *et al.*, 2008) and indirectly through the regulation of supaspinal pain control areas like the RVM (Pinto-Ribeiro *et al.*, 2008). However, the neuronal afferent and efferent pathways that might mediate nociceptive stimuli impinging on the PVN and its descending nociceptive modulatory actions, respectively, remain to be fully disclosed. Hence, the present study sought to map the detailed supraspinal brain centres connected with the PVN, including those that might participate in this process.

2. Material and methods

2.1. Animals

All experiments were performed in male Wistar-Han rats with 280-320g (Charles Rivers, Barcelona, Spain) according to the EEC guidelines (Directive 86/609/EEC) and the local veterinarian committee for the Handling of Laboratory Animals. Subjects were maintained under standard laboratory conditions at the animal facilities of the Life and Health Science School, University of Minho.

2.2 Retrograde-tracing study

In order to determine the brain nuclei projecting to the different areas of the PVN, 20 adult male rats (280–320g) were injected unilaterally with the retrograde tracer Cholera Toxin subunit B (CTb; List Biological Laboratories, Campbell, CA). Animals were anaesthetized with pentobarbitalsodium (50mg/kg i.p.) and placed in the stereotaxic frame (Stoeling; Chicago, IL) where the scalp was exposed through am incision on the midline of the scalp. A small hole, caudal to bregma, was drilled to allow the placement of a glass micropipette (20-30 μm diameter) into the brain. An injection of 1% CTb was administered iontophoretically into the right PVN using a positive DC current of 2.5μA for 5-10min (5s ON/5s OFF) according to the stereotaxic parameters of Paxinos & Watson (2005) (-1.72 mm caudal to bregma, +0.2 mm lateral to midline; -8.0 mm dorso-ventral). After completion of the injection the micropipette was

left *in situ* for 10-15 min before being slowly retracted to avoid reflux of the tracer through the micropipette tract. One-two weeks later, the animals were reanaesthetized with 35% chloral hydrate (1mL/kg body weight) and rapidly perfused transcardially with 100 ml buffered saline phosphate (PBS) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The perfused brains were removed from the animals and postfixed with the same fixative for 2h followed by 8% sucrose in 0.1 M PBS at 4°C until further treatment. Before sectioning, the contralateral part of the brain was marked; coronal sections of the whole brain of each animal were then cut at 50 µm using a vibratome. One in every three brain sections was immunoreacted for CTb and counterstained using the formol-thionin technique (Donovick, 1974) and one in every three section was only immunoreacted for CTb. The immunohistochemical procedures used were previously described (Almeida & Lima, 1997; Almeida *et al.*, 2000). Briefly, free-floating sections of the brain were immersed in a solution containing PBS with 0.3% Triton X-100 (PBST) and 1.5% of H2O2 over 15 min in order to inactivate the endogenous peroxidase activity. Sections were incubated overnight at room temperature in a goat primary polyclonal antibody against CTb (List Biological Laboratories, USA, 1:40.000 dilution in 0.3% PBST). The sections were washed twice in PBST, and they were incubated for 1 h in a 1:200 solution of a biotinylated horse anti-goat antibody (Vector Laboratories, Burlingame, USA). The sections were washed with PBST and then with 0.1M tris-HCl, pH8.2, and incubated for 1 h at room temperature in PBST containing an avidin-biotin complex (ABC, 1:200, Vector Laboratories, Burlingame, USA). Peroxidase was revealed using 0.0125% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Immunochemicals, St. Louis, USA) and 0.025% H2O2 in the same buffer. The sections were washed, dehydrated and placed on SuperFrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany) and mounted in Entellan (Merck, Darmstadt, Germany). In order to determine whether the injection was restricted to the PVN, a series of injections were performed in control sites. The same procedure as above was used to remove, preserve and develop these brains. Furthermore, on the remaining set of sections, control experiments for each experiment were done to determine whether the primary or the secondary antibodies produced false-positive results. In these experiments, sections were stained with all possible combinations of primary and secondary antibodies in which a single immunoprobe was omitted. Omission of primary or secondary antibodies resulted in the absence of labeling, demonstrating that no false-positive results were obtained with these reagents.

2.3. Anterograde-tracing study

Ten rats received iontophoretic injections (as above) of 10% biotinylated dextran-amine (BDA; 10,000 MW; Vector Laboratories, Burlingame, USA) in the right PVN through glass micropipettes (as above). After the completion of the injection period the micropipettes were left in place for 1o-15 min before being slowing retracted. Two to three weeks later, animals were reanaesthetized and perfused as described above. The entire brain was removed and prepared as above. Coronal sections of the entire brain were serially cut on a vibratome at 50 μm and the endogenous peroxiade activity was blocked as above. Two in every three successive brain sections were immunoreacted with ABC (1:200) for 1 h and then BDA was revealed with 0.0125% DAB and 0.02% H_2O_2 in Tris-HCl buffer 0.05 M, pH 7.6. Half of these sections were counterstained using the formol-Thionin technique and the remaining were left without any counterstaining. Sections were then serially placed in SuperFrost Plus slides, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany). Some injections were performed around the PVN in order to confirm the labeling of injections restricted to the PVN. In a few sections, omission of ABC resulted in absence of labeled fibres in the brain.

2.4. Data analysis

Cytoarchitectonic diagrams of the injection sites were made using the aid of a camera lucida. Retrogradely and anterogradely labeled neurons and fibres, respectively, in brain areas connected with the PVN were identified and superimposed on coronal representations of the rat brain (Paxinos and Watson, 2005), following the technique of Leite-Almeida and Almeida (2007). All sections were observed under a light microscope (Leica), photographed and identified. In all animals and sections, neurons and fibres were counted bilaterally along the rostrocaudal axis according to the stereotaxic coordinates of the atlas of Paxinos and Watson (2005). In **Table 1** is discriminated the number of neurones detected along the brain, whereas the presence of fibres were taking into account not only the amount of fibres with labeled terminals but also its consistency throughout successive serial sections where a given nucleus is present. The nomenclature/abbreviations used to designate brain nuclei and fibre tracts, except for a few exceptions, are in accordance with those used by Paxinos and Watson (2005).

3. Results

3.1. Injection sites

Amongst the fifteen CTb injections, three were restricted to the parvocellular part of the PVN, three comprised the parvocellular and magnocellular part of the PVN, one was on the magnocellular part of the PVN (**Fig. 1A**), two were located in the parvocellular part of the PVN and the periventricular nucleus, one was strictly on the periventricular nucleus. As control injections, one was placed dorsally to the parvocellular part of the PVN; one was located laterally to the PVN (**Fig. 1B**); one was ventral to the magnocellular part of the PVN and two showed no traces of injection. Ten animals were injected with BDA, two injections were outside the PVN (**Fig. 2B**) and of the remaining eight, five injections were located within the magnocellular PVN (**Fig. 2A**), two in the parvocellular part and one in the periventricular nucleus. The data presented in this study represents results from injections that were strictly within the anatomical borders of the PVN.

3.2. Retrograde labeling

CTb-labeled neurons were distributed along the rostrocaudal extension of the brain, but mainly in the telencephalon and diencephalon. CTb expression, although bilateral, was predominantly ipsilateral to the site of the injection. Analysis of CTb-containing neurons showed that they were present mainly in the telencephalon area, followed by the diencephalon and then the brainstem. A systematic analysis of the location and density of neurones projecting to the PVN is described in Table 1.

3.2.1. Telencephalon

The most prominent projections from the telencephalon to the PVN were observed from the dorsal tenia tecta (DTT) (bilateral), the intermediate part of the lateral septal nucleus (LSI) (bilateral) and the ventral lateral septal nucleus (LSV) (ipsilateral) (**Fig. 3A-F**). An ipsilateral projection to the PVN was shown to occur from the anterior and posterior parts of the anterior olfactory nucleus (AOM and AOP), the core of the nucleus accumbens (AcbC), the caudate-putamen (Cpu), the islands of Calleja (ICj and ICjM) and the cingulated (Cg1 and Cg2) cortex. Bilateral projections to the PVN occurred from the dorsal endopiriform nucleus (Den), the prelimbic (PrL), the infralimbic cortex (IL), the dorsal peduncular cortex (DP), the shell of the nucleus accumbens (AcbSh), the adrenaline cells (C1) and the lateral stripe of the striatum (LSS). In the amygdala all the projecting neurones were exclusively located ipsilaterally through various subnuclei, such as the medial and lateral divisions of the central amygdaloid nucleus (CeM and CeL), the posterior and anterior basolateral and basomedial part of the amygdaloid

nucleus (BLP and BLA), in the subiculum (VS) (**Fig. 4B**), the main part of the intercalated amygdaloid nucleus (IM and the of the lateral amygdaloid nucleus (LaVL). More ventrally we observed a few scattered labelled neurones in the medial and posterior part of the basomedial amygdaloid nucleus (BMA and BMP), the posterodorsal and ventral parts of the medial amygdaloid nucleus (MePD and MePV), the posteromedial part of the amygdalohippocampal area (AHiPM), the amygdalopiriform transition area (APir) the posterolateral and posteromedial cortical amygdaloid nucleus (PLCo and PMCo) and the bed nucleus of the accessory olfactory nucleus (BAOT). Some neurones were also observed in the ipsilateral side of the substantia innominata (SIB). A smaller number of bilaterally labelled neurones were scattered along the lambdoid septal zone (Ld), the medial septal nucleus (MS) and the median forebrain bundle (mfbb). On the other hand, a massive number of bilaterally labelled neurones were located in the various subnuclei of the stria terminalis (BSTMA, BSTL, BSTLV, BSTMV, BSTMPi, BSTMLi) (**Fig. 4A**). In the anterior part of the anterior commissure (aca) a few neurones were labelled bilaterally, whereas from the ventral palladium (VP) the projections were exclusively ipsilateral.

3.2.2. Diencephalon

In the diencephalon, a large number of neurones were distributed along several hypothalamic nuclei (**Fig. 3B-F**). In the preoptic region, the anterodorsal preoptic nucleus (ADP), the parastrial nucleus (PS) and ventrolateral part of the preoptic nucleus (VLPO) were the main PVN-projecting nuclei. Projections from the medial preoptic arca (MPA), the medial, central and lateral parts of the medial preoptic nucleus (MPOM, MPOC and MPOL), the posterodorsal preoptic nucleus (PDP), the ventromedial part of the preoptic nucleus (VMPO), the supraoptic nucleus (SO), the lateral preoptic area (LPO), the suprachiasmatic nucleus (Sch) and the retrochiasmatic area (Rch) were all bilateral. In the anterior region of the hypothalamus the anterior parvicellular and medial and the ventral part of the paraventricular hypothalamic nucleus (PAaP, PaMP and PaV), the anteroventral periventricular nucleus (AVPe), periventricular hypothalamus nucleus (Pe) and the the medial corticohypothalamic tract (mch) presented bilateral staining. Ipsilateral projections were observed only from the central part of the anterior and posterior hypothalamic area (AHC and AHP) and the striohypothalamic nucleus (StHy). In the tuberal region of the hypothalamus a moderate number of retrogradelly ipsilateral labelled neurones were found and only a few were located contralaterally. The nuclei projecting to the PVN were the compact part of the dorsomedial hypothalamic nucleus (DMC), the dorsal part and compact part of the dorsomedial

hypothalamic nucleus (DMV and DMD) the dorsomedial and the ventrolateral part of the ventromedial hypothalamic nucleus (VMHDM and VMHVL), the arcuate nucleus (ArcLP, ArcMP, ArcD and ArcM) the posterior hypothalamic area (PH), the tuber cinerum (TC) and the medial tuberal nucleus (MTu). In the posterior region of the hypothalamus projections were bilateral from the mammillary recess of the 3rd ventricule (MRe) and the ventral and dorsal tuberomammillary nucleus (VTM and DTM). In the thalamus, many nuclei presented numerous CTb-labelled neurones, mainly ipsilaterally, such as the posterior part of paraventricular thalamic nucleus (PVP), the medial part of the mediodorsal thalamic nucleus (MDM), the parvicellular part of the ventral posterior thalamic nucleus (VPPC) and the ventral reunions thalamic nucleus (VRe), the dopaminergic cells (A11), the ventral part of the zona incerta (ZIV) and the periventricular fiber system (pv). More laterally, the posterior intralaminar thalamic nucleus (PIL) and the triangular part of the posterior thalamic nuclear group (PoT) were strongly labelled.

3.2.3. Mesencephalon

In the mesencephalon the most proeminent projections originated from the periaqueductal gray matter (PAG), namely the dorsolateral and dorsomedial parts (DLPAG and DMPAG), the posterior intralaminar thalamic nucleus (PIL) and from the retrorubral field (RRF) (**Fig. 3G,H**). Other areas presented a small number of scattered labelled neurones, like the central tegmental tract (ctg), posterior thalamic nuclear group (PoT) and the commissure of the superior colliculus (csc). A few labelled neurones were observed in the nigrostriatal bundle (ns), the rostral linear nucleus of the raphe (RLi), the red nucleus (RPC), the peripeduncular nucleus (PP), the marginal zone of the medial geniculate (MZMG), the superior cerebellar peduncule (scp), the pararubral nucleus (PaR), the deep mesencephalic nucleus (DPMe), the medial and lateral parts of the medial mammillary nucleus (MM and ML) the caudal, dorsolateral, intermediate and rostral subnucleus of the interpeduncular nucleus (IPC, IPDL, IPI and IPR), the ventral tegmental arca (VTA) and the reticular part of the substancia nigra (SNR).

3.2.4. Pons and Medulla Oblongata

Projections originating in the pons and medulla were modest and mostly from the ipsilateral side (**Fig. 3I-K**). The areas with greater projections were the gigantocellular reticular nucleus (Gi), the perifacial zone (P7) and the caudal ventrolateral medulla (CVLM) (**Fig. 4D**). A small number of scattered stained neurones were found in reticular nuclei (parvicellular nucleus), intermediate

parts of the facial [dorsal intermediate (7L), dorsomedial (7DM) and ventral intermediate (7VI)] and prerubral nuclei (Pr). A few labelled neurones were observed in the lateral paragigantocellular (LPGiA), the spinal trigeminal (Sp5O), the medullary dorsal reticular nucleus (DRt) (**Fig. 4C**) and the superior salivatory (SuS).

3.3. Anterograde labeling

BDA-stained fibres were distributed along the rostrocaudal extension of the brain, but mainly in the telencephalon area, followed by the diencephalon and then the brainstem. BDA expression, although bilateral, was predominantly ipsilateral to the site of the injection. A systematic analysis of the location and density of areas targeted by the PVN is described in Table 1.

3.3.1. Telencephalon

The most prominent PVN projections to the telencephalon included the agranular insular cortex (AIV) (**Fig. 5C**), the ventral palladium (VP) (bilateral), the lateral orbital cortex (Lo), the dorsal endopiriform nucleus (DEn), the caudate putamen (CPu) (bilateral) (**Fig. 3A-F**) and the intermediate (bilateral) and posteromedial part of the medial division of the bed nucleus of the stria terminalis (STLi and STMPM, respectively). Bilateral fibres were also observed in the core and shell of the nucleus accumbens (AcbC and AcbSh, respectively), the olfactory tubercle (Tu1), the dorsal part of the substancia innominata (SIB), the septohippocampal nucleus (Shi), the secondary Motor Cortex (M2), the lateral entorhinal cortex (LEnt), the nucleus horizontal limb of the diagonal band (HDB) and the cingulated cortex (Cg1). Ipsilaterally stained fibres could be identified in the ventral orbital cortex (VO), the ventral endopiriform nucleus (VEn), the ventral part of claustrum (VCl), the mediomedial area of the secondary visual cortex (V2MM), the prelimbic, infralimbic, agranular and dysgranular insular cortices (PrL, IL, AIP and DI, respectively) (**Fig. 5B**), the lateral accumbens shell (LAcbSh), the sublenticular and central amygdalar nuclei (EAC and CeE, respectively), the ventral striatum (CB) and the adrenaline cells (C1). Scattered ipsilateral fibres were also observed in the mediolateral and lateral areas of the secondary visual cortex (V2ML and V2L, respectively), the triangular septal nucleus (TS), the temporal association, secondary somatosensory and retrosplenial granular and dysgranular and perirhinal cortices (TeA, S2, RSGc, RSD and PRh, respectively), the paracommissural nucleus (PaC), the navicular nucleus of the basal forebrain (Nv), the medial septal nucleus (MS), the posterodorsal and anteriordorsal medial amygdaloid nuclei (MeAD and MePD, respectively), the

ventrolateral part of the lateral amygdaloid nucleus (LaVL), the anterolateral part of the medial division of the bed nucleus of the stria terminalis (STMAL), the intermediate endopiriform nucleus (IEn), the dorsal peduncular cortex (DP), the dorsal part of the claustrum (DCl), the posterior, anterior and ventral parts of the basomedial amygdaloid nucleus (BMP, BMA and BLV, respectively), the basal nucleus (B), the posterior part of the anterior olfactory nucleus (AOP), the anterior cortical amygdaloid nucleus (ACo), the rostral pole of the nucleus accumbens (AcbR) and the anterior amygdaloid area (AA). A few bilateral fibres could be observed in the primary motor cortex (M1), the lateral and lambdoid septal zone (LSI and Ld, respectively), the medial part of the sublenticular extended amygdala (EAM), the anteromedial part of the medial division of the bed nucleus of the stria terminalis (STMAM), the cingulated cortex (Cg2) and the amygdalopiriform transition area (APir).

3.3.2. Diencephalon

In the diencephalon, strong bilateral efferent output from the PVN targeted mainly the hypothalamus followed by a modest but consistent output to thalamic areas (**Fig. 3B-F**). The areas with strongest bilateral stained fibres were the compact and dorsal areas of the dorsomedial hypothalamic nucleus (DMC and DMD, respectively), in the dorsal hypothalamic area (DA), in the dorsomedial part of the ventromedial hypothalamic nucleus (VMHDM), in tuberal region of lateral hypothalamus (TuLH) and in the medial part of the medial preoptic nucleus (MPOM), in the juxtaparaventricular lateral hypothalamus (JPLH), in the submedius and mediodorsal thalamic nucleus, dorsal part (SubD and MDC, respectively)and the accessory neurosecretory nuclei (ANS). Ipsilateral strong staining was also mainly, but not exclusively, found in hypothalamic areas such as in the ventral dorsomedial, central ventromedial and lateroanterior hypothalamic nuclei (DMV, VMHC and LA, respectively), in the dorsal posterior and anterior hypothalamic areas (PHD and AHA, respectively) and the peduncular part of lateral hypothalamus (PLH). Other areas with strong ipsilateral staining comprise the ventral and dorsal parts of the zona incerta (ZIV and ZID), the parastrial nucleus (PS) and the anteroventral thalamic nucleus (AVD). Within the thalamus bilateral but modest projections could be found in the posterior paraventricular, the medial and lateral parts of the mediodorsal, intermediodorsal, the ventral posteromedial and posterolateral, the paracentral, the interanterodorsal thalamic nucleus, the central medial, ventral part of the submedius and in the reunions thalamic nuclei (PVP, MDM, MDL, IMD, VMP, VPL, PC, IAD, CM, SubV and Re, respectively), in the perifornical part of lateral

hypothalamus (PeFLH), the lateral preoptic area (LPO) and in the A13 dopamine cells. Ipsilateral modest projections originated from the ventrolateral part of the laterodorsal and ventral part of the anteromedial thalamic nuclei (LDVL and AMV, respectively), the shell of the ventromedial and the ventrolateral hypothalamic nuclei (VMHSh and VLH, respectively), the lateral part of the medial preoptic, the medial and the lateral part of the habenular, the medial tuberal, the perifornical and the septohypothalamic nucleus (MPOL, MHb, LHbL, MTu, PeF and SHy, respectively), the nucleus of the stria medullaris (SM) and periventricular fiber system (Pv).

Scarce but bilateral projections target the reticular, the ventral reuniens and paratenial thalamic nuclei (Rt, VRe and PT, respectively), the retrochiasmatic and the medial preoptic areas (RCh and MPA) and the anteromedial part of the medial division of the bed nucleus of the stria terminalis. A few stained fibres were found in the anteromedial, anterodorsal, anterior paraventricular and rhomboid thalamic nuclei (AD, AM, PVA and Rh, respectively), in the ventrolateral, the septofimbrial, the arcuate, the central part of the medial preoptic nuclei (VLPo, SFi, ArcM and MPOC, respectively), the primary sensory cortex (S1FL) and the ventral area of the secondary auditory cortex (AuV).

3.3.3. Mesencephalon

In the mesencephalon the most prominent projections are to the periaqueductal gray matter (PAG), namely the ventrolateral, dorsolateral and lateral parts (VLPAG, DLPAG and LPAG) (**Figure 5 A**) and from the laterodorsal and the pericentral part of the dorsal tegmental nuclei (LDTg and DTgP, respectively) and the posterior commissure of the paracommissural nucleus (PaC) (**Figure 3 G,H**). Ipsilaterally labeled fibres could be found in the lateral part of the dorsal raphe nucleus (DrL) and the dorsomedial periaqueductal gray (DMPAG). A few bilateral labeled fibres were observed in the ventral and intermediate parts of the cuneiform nucleus (CnFV and CnFI, respectively), in the paramedian and median raphe and pararubral nuclei (PMnR, MnR and PaR, respectively), in the pedunculopontine tegmental nucleus (PPtg), the plioglial part of periaqueductai gray (PIPAG) and in the intermediate, deep white layer and deep gray layer of the superior culliculus (InWh, DpWH and DpG). Scarce ipsilateral fibres were observed in rhadboid and peripeduncular nuclei (RBP and PP, respectively), the dorsal tier of the substantia nigra (SNCD), the rostral linear nucleus of the raphe (RLi), the triangular part of the posterior thalamic nuclear group (PoT), the oral part of the pontine reticular nucleus (PnO), the paranigral nucleus of the VTA (PN), the posterior intralaminar thalamic nucleus (PIL), the nucleus of the optic tract

(OT), the marginal zone of the medial geniculate (MZMG), the ventral part of the laterodorsal tegmental nucleus (LDTgV), the dorsal part of the interpeduncular nucleus (IPDL), the intermediate gray layer superior colliculus (InG), the deep mesencephalic nucleus (DpMe), the dorsal and the caudal parts of the dorsal raphe nucleus (DrD and DRC) and the commissural nucleus of the inferior colliculus (com).

3.3.4. Pons and Medulla Oblongata

Considerable bilateral efferent projections from the PVN could only be found in the lateral paragigantocellular nucleus (LPGiA) and in the gigantocellular reticular nucleus (Gi) (**Figure 3 I-K**). Ipsilateral stained fibres were located mainly in the raphe pallidus and raphe magnus nuclei (RPa and RMg, respectively) and in the intermediate, ventral and medial parts of the medullary reticular nucleus (IRtA, MdV and MdM, respectively). Contralateral scarce fibres were also detected in the medial lemniscus (ml) and in the alpha part of the gigantocellular reticular nucleus (GiA).

4. Discussion

4.1. Technical considerations

Histologically, the parvocellular division of the paraventricular nucleus of the hypothalamus can be delimitated laterally from the magnocellular division of the PVN, ventrally from the periventricular nucleus of the hypothalamus and dorsally from the medial corticohypothalamic tract (mch). In this study, we employed the CTb tracer because it produces an intense retrograde labelling even when applied through small iontophoretic injections (Ericsson and Blomqvist, 1988; Almeida *et al.*, 2002). Four possible sources of uncertainty inherent to the use of tract-tracing technique were taken into account in the interpretation of the present findings. Firstly, the small iontophoretic CTb injections resulted in restricted and perfectly defined injection sites, all located within the boundaries of the parvocellular division. Secondly, no leakage of CTb was detected after the micropipette track was examined; this was avoided due to the use of micropipettes with small diameter tips and to the time allowed for the micropipette to remain in the injection place before retraction. Thirdly, the possibility of the CTb being trans-synaptically transported was ruled out as it was shown by Almeida *et al* (1993). Fourthly, the risk that CTb may retrogradely label some brain areas because of its uptake by passing fibres, however this

possibility was also refuted by others (Lima *et al.*, 1991). Therefore, we concluded that these injections resulted exclusively in CTb retrograde labelling of brain neurones with axons terminating in the parvocellular division of the PVN. On the other hand, BDA is also a powerful anterograde tracer (Novikov, 2001). The specificity of the labeling resulting from BDA has been confirmed by injecting slightly different portions of the the DRt (Almeida *et al.*, 1995) or by control injections in nuclei just bordering the area of interest (Leite-Almeida *et al.*, 2006) and showing that these resulted in completely different patterns of brain projections. However, BDA can be incorporated into injured dendrites and fibres at the injection site. The potential labeling of passing fibres can be almost abolished by using small diameter micropipette tips during the iontophoretic process. This was the case of the present and previous (Leite-Almeida *et al.*, 2006) studies since virtually no labeled perikarya were found along the brain of injected animals. Finally, the intensity of fibre staining in the mesencephalic, pons and medulla oblongata was rather scarce in this study when compared to a recent work (Gueerling *et al.*, 2009). One explanation could be the use of different anterograde tracers [BDA vs. PHAL (*Phaseolus vulgaris* leucoagglutinin)], it is however not probable since both tracers are widely used in anatomical studies. Alternatively, this fact is probably related a difference in the amount of tracer injected in each case, since in our study BDA injections are much smaller than the PHAL injections performed by Gueerling and colleagues (2009).

4.2. Brain connections to the PVN: anatomical considerations

This is the first work dedicated to the systematic evaluation of the forebrain and mesencephalic areas connected to the PVN, through direct anterograde and retrograde tracer iontophoretic microinjection to this area, with enphasis on its role in pain modulation.

4.2.1. Telencephalon

To the best of our knowledge the projections from the prelimbic cortex, the islands Calleja, the caudate-putamen, the cingulated cortex, the bed nucleus of the accessory olfactory nucleus, the substantia innominata and the ventral palladium to the PVN were reported here for the first time. Several previous studies have mentioned functional relationships between the caudate-putamen, the cingulated cortex, the bed nucleus of the accessory olfactory nucleus and the substantia innominata however a direct anatomical pathway has never been described (Broad *et al.*, 1993; Karimnamazi & Travers, 1998; Bourgeais *et al.*, 2001). Projections from the shell and core of the

nucleus accumbens were previously described by several authors (Bertolucci-D'Angio *et al.*, 1990; Florin *et al.*, 2000; Barrot *et al.*, 2002; Magnusson & Martin, 2002). Perez-Rosado and colleagues (2000) referred to projections to several brain nuclei from the caudate-putamen, but not specifically to the PVN; the same occurring with the cingulated cortex (Drossman *et al.*, 2003; Hart *et al.*, 2003; Meller *et al.*, 2003). Bilateral projections were shown from the dorsal endopiriform nucleus (Beck & Fibiger, 1995; Florin *et al.*, 2000) and the adrenaline cells (Palkovits *et al.*, 1999). Labeled cells were found in the amygdala, primarily in the medial nucleus, although the number of projecting neurones was smaller than expected (Kalivas *et al.*, 1982; Oldfield & Silverman, 1985; Bon *et al.*, 1998; Bourgeais *et al.*, 2001; Michl *et al.*, 2001; Alheid, 2003). The amount of labeled neurones in the dorsal tenia tecta (Piekut & Phipps, 1998; Weitemier *et al.*, 2001) the intermediate and ventral part of the lateral septal nucleus (McNally & Akil, 2002) was considerably higher than what is found in previous works. In what concerns the PVN projections, they terminate mainly in the ipsilateral agranular insular cortex, nucleus accumbens, amygdala and caudate-putamen, areas that to the best of our knowledge are referred here for the first time.

4.2.2. Diencephalon

To the best of our knowledge, the projections from the tuber cinerum and the striohypothalamic nucleus were reported here for the first time. Projections originated in other diencephalic nuclei have since long been illustrated although the data is often fragmented. As Garris (1979) described, labeled neurones were found in the anterior diencephalon scattered along the septal zone and nuclei. The stria terminalis is the brain area that presented the largest number of projecting cells, which was expected due to its physiological functions, namely in stress and homeostasis (Bester *et al.*, 1997; Van de Kar & Blair, 1999; Crown *et al.*, 2000; McNally & Akil, 2002) and corrobates observations by Dong and colleagues (2001). As Silverman and colleagues (1981) observed in the preoptic area and in the hypothalamus, the medial groups have larger input than do the lateral areas. Once again it was observed that the PVN receives input from the contralateral PVN (Silverman *et al.*, 1981). In the thalamus many nuclei presented intense labeled neurones mainly ipsilateral, but none of them showed a consistent number of labeled cells. Major projections from the PVN were present in other hypothalamic nuclei, medial preoptic nucleus and thalamic nucleus submedius.

4.2.3. Mesencephalon

To the best of our knowledge the projections from the retrorubral field, the nigrostriatal bundle, the superior cerebellar peduncule and the pararubral nucleus was reported here for the first time. Projections of the central tegmental tract to the PVN have been reported previously (Leibowitz & Brown, 1980) as were projections from the commissure of the superior colliculus (Morcuende *et al.*, 2002; Tardif & Clarke, 2002), the deep mesencephalic nucleus (Meloni and Davis, 1999; Rodriguez *et al.*, 2001; Gonzalez-Hernandez *et al.*, 2002), the medial mammillary nucleus (Mihaly *et al.*, 2002; Timofeeva *et al.*, 2003), the tegmental area (Gueerling *et al.*, 2009) and the interpeduncular nucleus (Rivest *et al.*, 1995; Salminen *et al.*, 1996; 2000). Concerning PVN afferent, the main target of the nucleus is clearly the PAG.

4.2.4. Pons and Medulla Oblongata

Both the retrograde and anterograde labeling from the PVN is modest or scarce. Note the absence of pain control centres projecting to the PVN, although a small PVN descending projection targets RVM regions and the DRt (Almeida *et al.*, 2006).

4.3. Functional considerations

Given the wide neuronal network of the PVN, the analysis of possible functional implications of these connections was artificially separated by systems and a greater emphasis was attributed to connections that may directly contribute to pain and stress modulation. Nonetheless, important projections between the PVN and areas involved in the limbic, autonomic and motor system were also addressed although to a smaller extent.

4.3.1. The supraspinal pain modulatory system

Our results demonstrate that chief supraspinal nuclei involved in the pain modulatory system share extense and reciprocal connections with the PVN. The IL, PrL and Cg regions of the prefrontal cortex display increased expression of c-Fos after exposure to several forms of stress (Ostrander *et al.*, 2003; Morrow *et al.*, 2000; Duncan *et al.*, 1996) reflecting the involvement of these areas in the modulation of the endocrine and autonomic response to stress (Herman *et al.*, 2005; Sullivan, 2004). The PrL has been demonstrated to be activated by fear, playing an essential role in the expression of learned fears (Corcoran and Quirk, 2007; Choi *et al.*, 2010). The existence of bilateral connections between the PVN and the PrL could partially account for the modulatory effect of fear upon pain perception (Holmes and Wellman, 2009). Also interesting

is the fact that the PrL regulates the expression of fear memories through projections to the AMY (Holmes and Wellman, 2009), an area that modulates the emotional dimension of pain. On the other hand, an excitatory role has been proposed for the IL, it increases stress-induced HPA axis activation, resulting in increased levels of plasma ACTH and corticosterone (Radley *et al.*, 2006). Curiously, inescapable stress elicits the release of serotonin from the PFC (Grahn *et al.*, 1999; Maswood *et al.*, 1998) and a large number of neurones in the IL and PrL areas project to RVM (Gabbott *et al.*, 2005). Additionally, the inactivation of the IL and PL results in stress-induced learned helplessness (Amat *et al.*, 2005), which contributes maladaptive responses to stress (Maier *et al.*, 2006; Maier and Watkins, 2005). The anatomical connections of the PFC with the PVN and HPA axis activity and the evidence of serotonergic projections from this area to the RVM could contribute, to some extent, to the analgesia induced by chronic unpredictable stress (Pinto-Ribeiro *et al.*, 2004) and to the role of serotonergic pathways in nociceptive processing by the PVN in normal and chronic nociceptive pain conditions (Pinto-Ribeiro *et al.*, 2008).

Recent studies have suggested that the BNST participates in the physiological manifestation of the emotional dimension of pain as part of the extended amygdala (Alheid *et al.*, 1995) and c-Fos immunoreactivity increased in the BST in response to noxious mechanical stimulation (Morano *et al.*, 2008). Moreover, the BNST areas activated by acute pain in normal animals were different from the ones activated by the same stimulus in animals suffering from neuropathic pain (Morano *et al.*, 2008). Choi and colleagues (2007) demonstrated that the posterior BNST nuclei mainly inhibit the HPA axis while the anteroventral BNST nuclei enhance its activity. The bilateral projections shared by the BNST and the PVN further substantiate the existence of a fine tuning of this area upon the neuroendocrine and autonomic emotional responses (LeDoux, 2000) to stress and pain.

As the PVN mediates the stress response it is expected that it shares intense reciprocal projections with the DMH, an area involved in defensive responses to threatening stressors (Fuchs and Siegel, 1984; Dielenberg *et al.*, 2001). Additionally, the DMH is strongly activated after swim and immobilization stress (Cullinan *et al.*, 1995) and contributes to anxiolytic states (Inglefield *et al.*, 1994), which also result in the activation of the HPA axis. The activation of these areas, which appear to counteract each other by inhibiting (Pinto-Ribeiro *et al.*, 2008) or facilitating (Pinto-Ribeiro et al., data not published; Martensen et al., 2009) nociception, respectively. The activation of these parallel pain modulatory pathways probably depends on

specific emotional clues since both areas receive projections from the PFC, the bed nucleus of the stria terminalis and the AMY (Geerling *et al.*, 2008; Thompson and Swanson, 1998) and are involved in the response to stress.

The thalamic PVP not only shares bilateral connections with the PVN but is also known to project to the PFC (Hurley *et al.*, 1991) while being target by fear and power-dominance motivation areas from the hypothalamus (Sewards and Sewards, 2002) and the lateral and dorsolateral PAG (Krout and Loewy, 2001), the AMY (Canteras *et al.*, 1995) and the BNST (Numan and Numan, 1996). In addition, the activation of the PVP can be elicited by fear and stress (Cullinan *et al.*, 1995; Campeau *et al.*, 1997). Sewards and Sewards (2002) proposed that the power-dominance drive is related to the rostral part while fear is connected with the posterior part of the PVP. It seems likely that this PVP-PVN might play an important part in the reinforcement of the motivational component of pain modulation by stress.

Reciprocal projections between the PVN and the PAG, an area that plays a crucial role in the integration of reactions to threatening or stressful events (Bandler *et al.*, 1991; Cullinan *et al.*, 1995), were restricted to the lateral, dorsolateral and ventrolateral areas. LPAG and DLPAG electric stimulation has been shown to produce a strong affective/defense as well as motor response (Bandler, 1982), identical to what is observed after exposure to predators. Also important is the fact that DLPAG is activated by audiogenic stress, which in turn activates the hypothalamo-pituitary- adrenals (HPA) axis in rats (Campeau *et al.*, 1997).

Reciprocal projections with the PnO are in line with previous works that proposed this nucleus not only has a mediator of hypothalamic stress-induced effects but also has a target for corticosteroid negative feedback effects (Li *et al.*, 1998).

In support of the antinociceptive action mediated directly upon the spinal cord and also with a contribution relaying in the RVM (Pinto-Ribeiro *et al*, 2008; Condés-Lara *et al*, 2009), the PVN is reciprocally connected with brain nuclei shown to be involved in antinociception, like the amygdala (Helmstetter and Bellgowan, 1993), basal ganglia (Chudler and Dong, 1995), the PAG (Basbaum and Fields, 1984) and deep mesencephalic nucleus (Wang *et al*, 1992) and projects to areas like the DRt (Lima and Almeida, 2002), the NTS and the CVLM (Tavares and Lima, 2002).

4.3.2. Other limbic-related connections

The PVN also seems to be implicated in the integration of stress response (cingulate cortex; Radley *et al.*, 2009), as the prefrontal influences on the HPA output are predominantly inhibitory and emanate from the prelimbic and/or dorsal anterior cingulated cortices. The reciprocal connections with the bed nucleus of the stria terminalis (BNST) points to an involvement in the tonic/unpredictable stress responses (Pêgo *et al.*, 2008). Reciprocal connections with the medial septal nucleus imply a possible participation of the PVN in cognitive performance (McKeon-O'Malley *et al.*, 2003) and avoidance behaviours (Riekkinen, 1994).

4.3.3. Other autonomic-related connections

Most of the forebrain and mesencephalic brain nuclei projecting to or receiving projections from the PVN belong to the autonomic control system. For that reason the PVN seems to be implicated in the immune response (islands Calleja; Engstrom *et al.*, 2003), the Alzheimer's disease (striohypothalamic nucleus; Mufson *et al.*, 1999), the generation and entrainment of several circadian rhythms (tuber cinerum; Mikkelsen, 1990), temperature, water and salt homeostasis (preoptic nucleus and periventricular nucleus; Sellami & de Beaurepaire, 1995; Baffi & Palkovits, 2000; Usdin *et al.*, 2000; Spitznagel *et al.*, 2001), the relay of convergent inputs from gustatory, baroreceptor, chemoreceptor organs (insular cortex; Hanamori *et al.*, 1998), blood pressure and cardiovascular function (ventral palladium; Allen & Cechetto, 1993) and reproduction (anteroventral periventricular nucleus; Salama *et al.*, 2003; Zup *et al.*, 2003).

4.3.4. Motor-related connections

The PVN seems to play a role in motor functions. Motor-related projections originating in the forebrain and brainstem regions were observed from the retrorubral field and facial nucleus, suggesting an involvement in the control of the orofacial musculature (Von Krosigk & Smith, 1991). Additionally the PVN projects largely to the basal ganglia and more modestly to the motor cortex.

5. Conclusion

In conclusion, the present results along with the literature available indicate that the PVN: (1) is implicated in a supraspinal network that integrates and / or information from autonomic, limbic, motor and antinociceptive systems; (2) is involved in an antinociceptive pathway, relaying the nociceptive information converging from pain-related nuclei in the forebrain, and; (3) is an

effector nucleus that triggers response actions in the systems involved in (1) and (2), due to its large descending projections to the mesencephalus and spinal cord (Ranson *et al.*, 1998).

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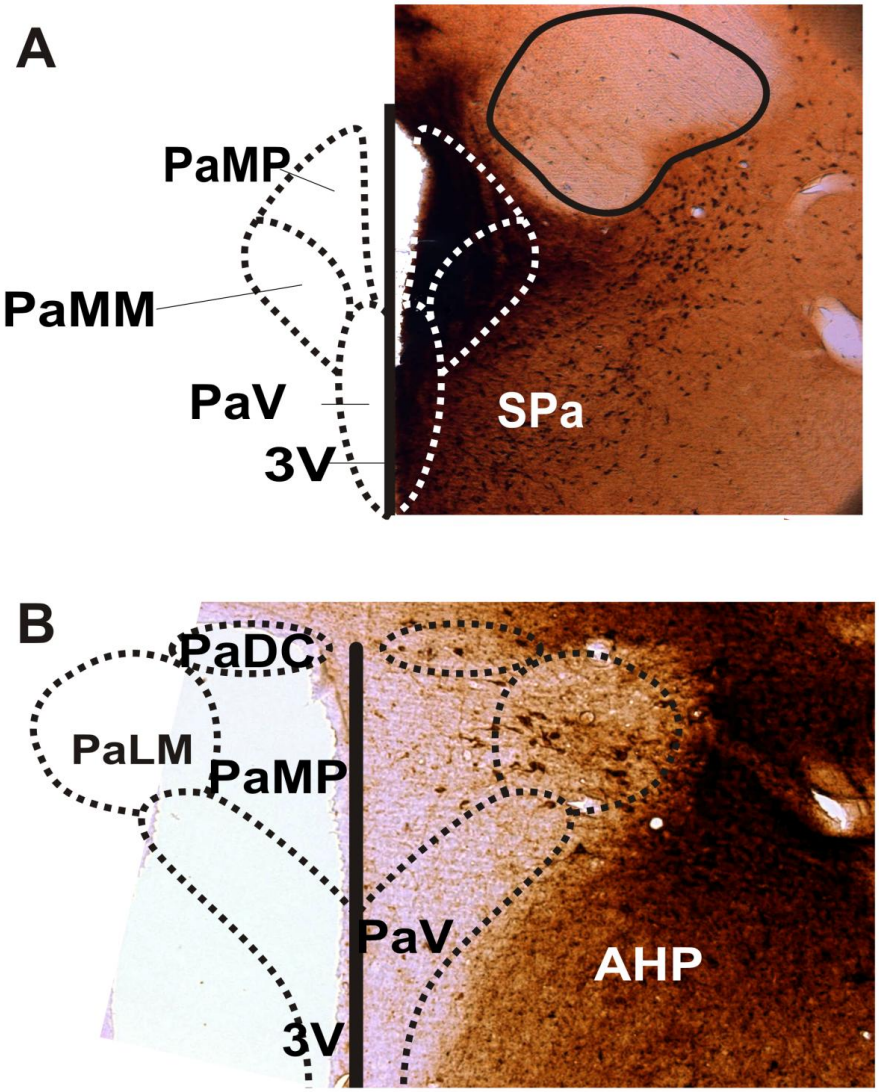


Figure 1 – Photomicrographs of CTb tracer and of a control injection sites in the paraventricular nucleus of the hypothalamus. A – CTb microinjection in the more rostral part of the PVN (RC: -1.44mm, LM: +0.62mm; DV: -7.9mm). B – Example of a control injection of CTb in an area immediately adjacent to the PVN (RC: -1.72mm, LM: +0.8mm; DV: -8.0mm). (RC - rostrocaudal coordinate; LM - lateromedial coordinate; DV- dorsoventral coordinate)

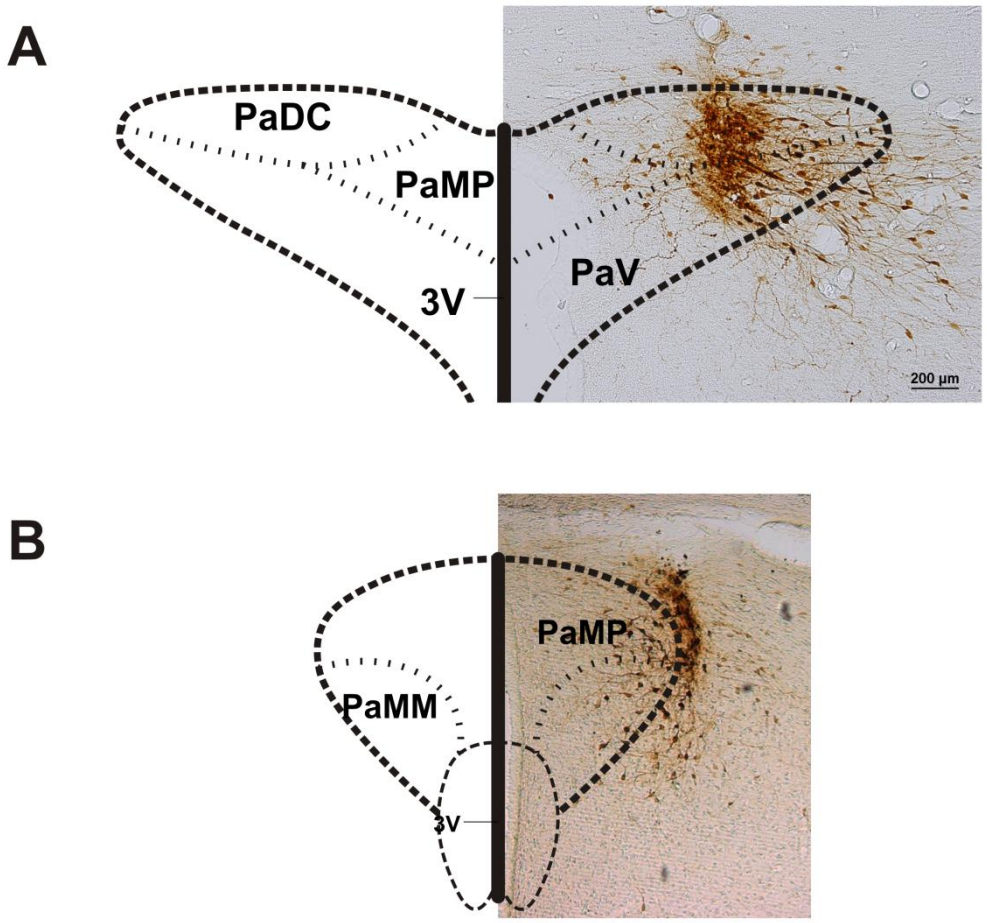


Figure 2 – Photomicrographs of BDA tracer and of a control injection sites at several coordinates of the paraventricular nucleus of the hypothalamus. A – Example of an injection site of BDA (RC: -1.92mm, LM: +0.6mm; DV: -7.9mm). B – Example of a control injection of BDA outside the lateral cap of the PVN (RC: -1.56mm, LM: +0.6mm; DV: -8.0mm). (RC - rostrocaudal coordinate; LM - lateromedial coordinate; DV- dorsoventral coordinate)

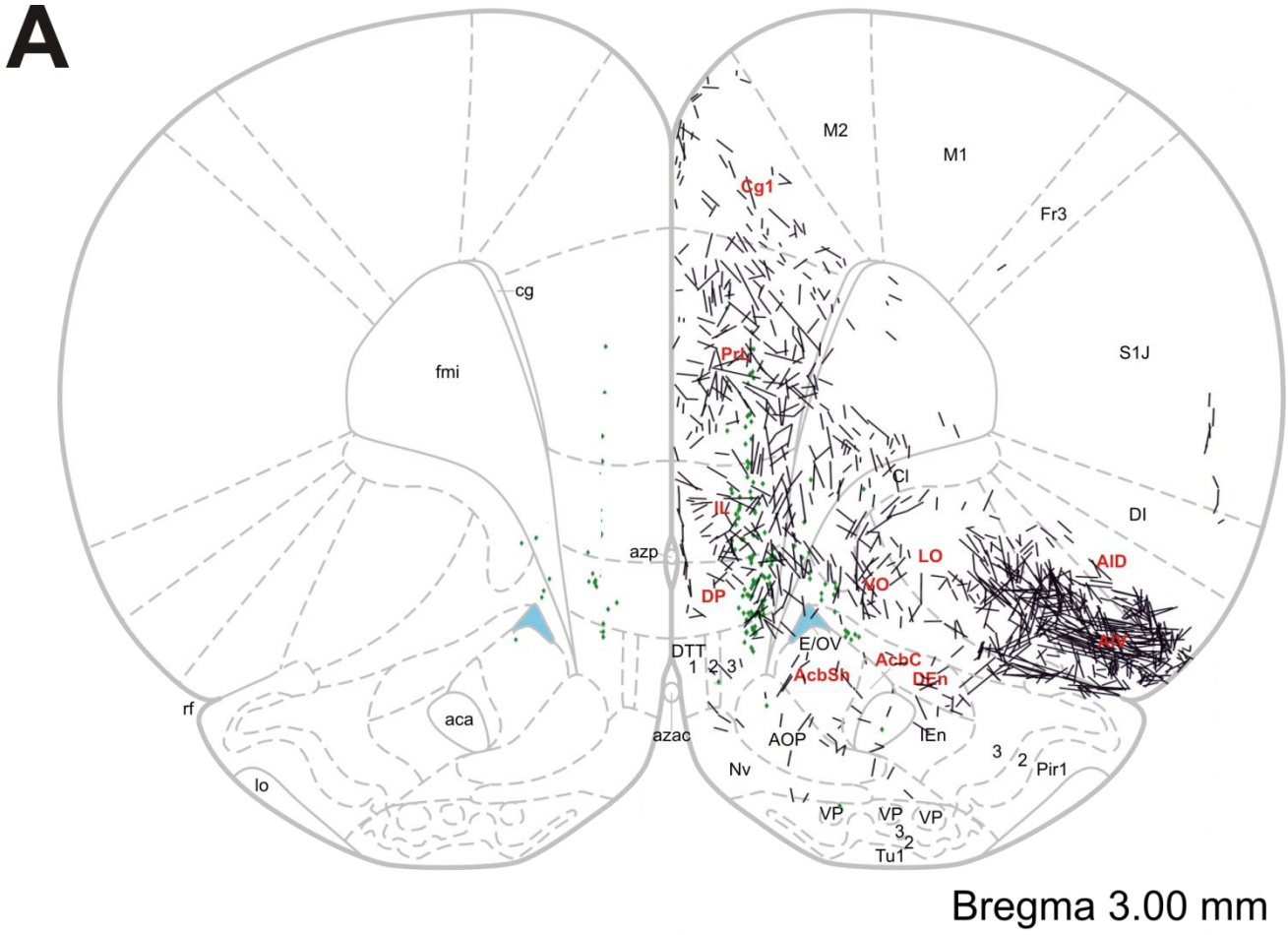
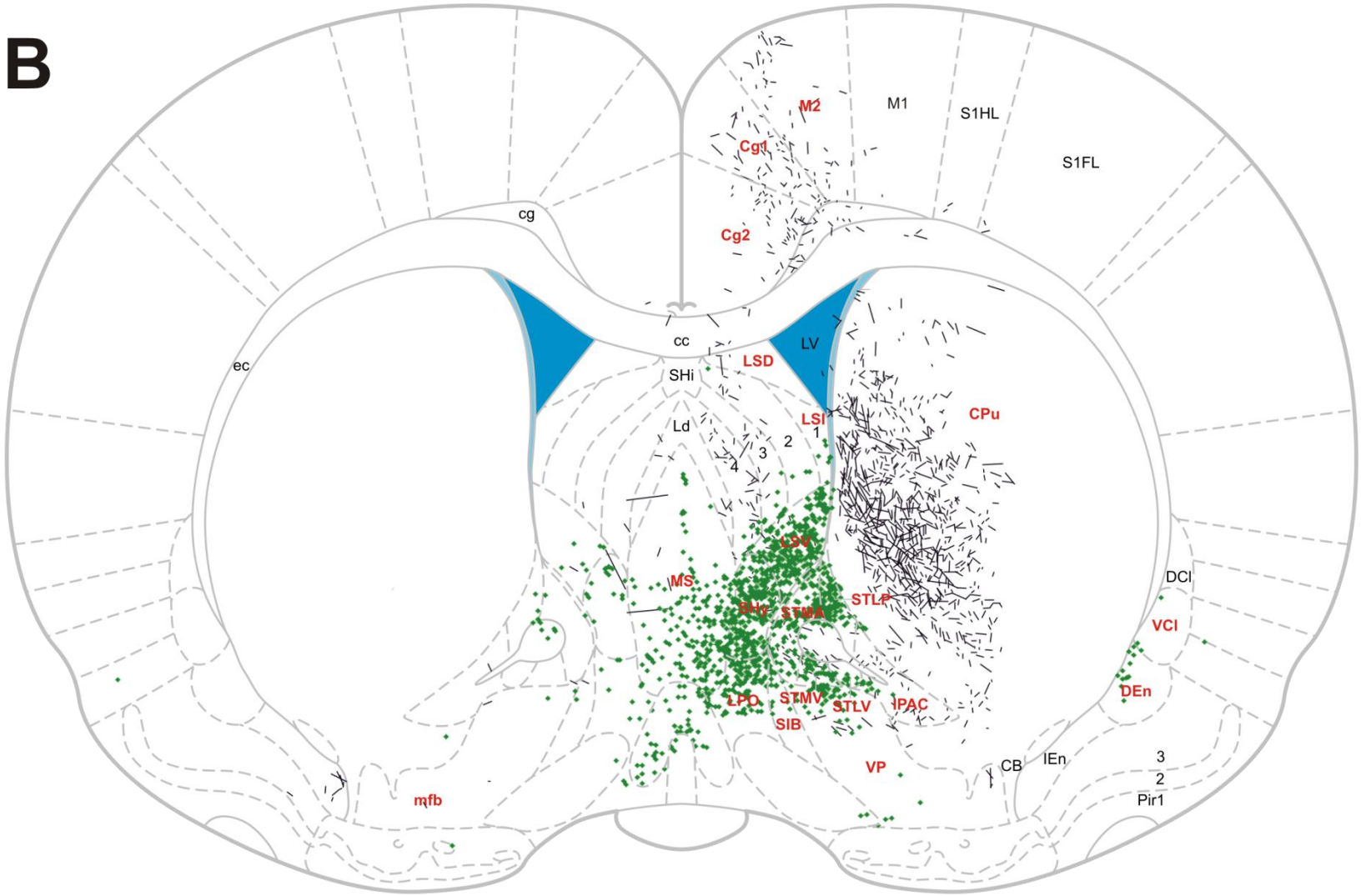
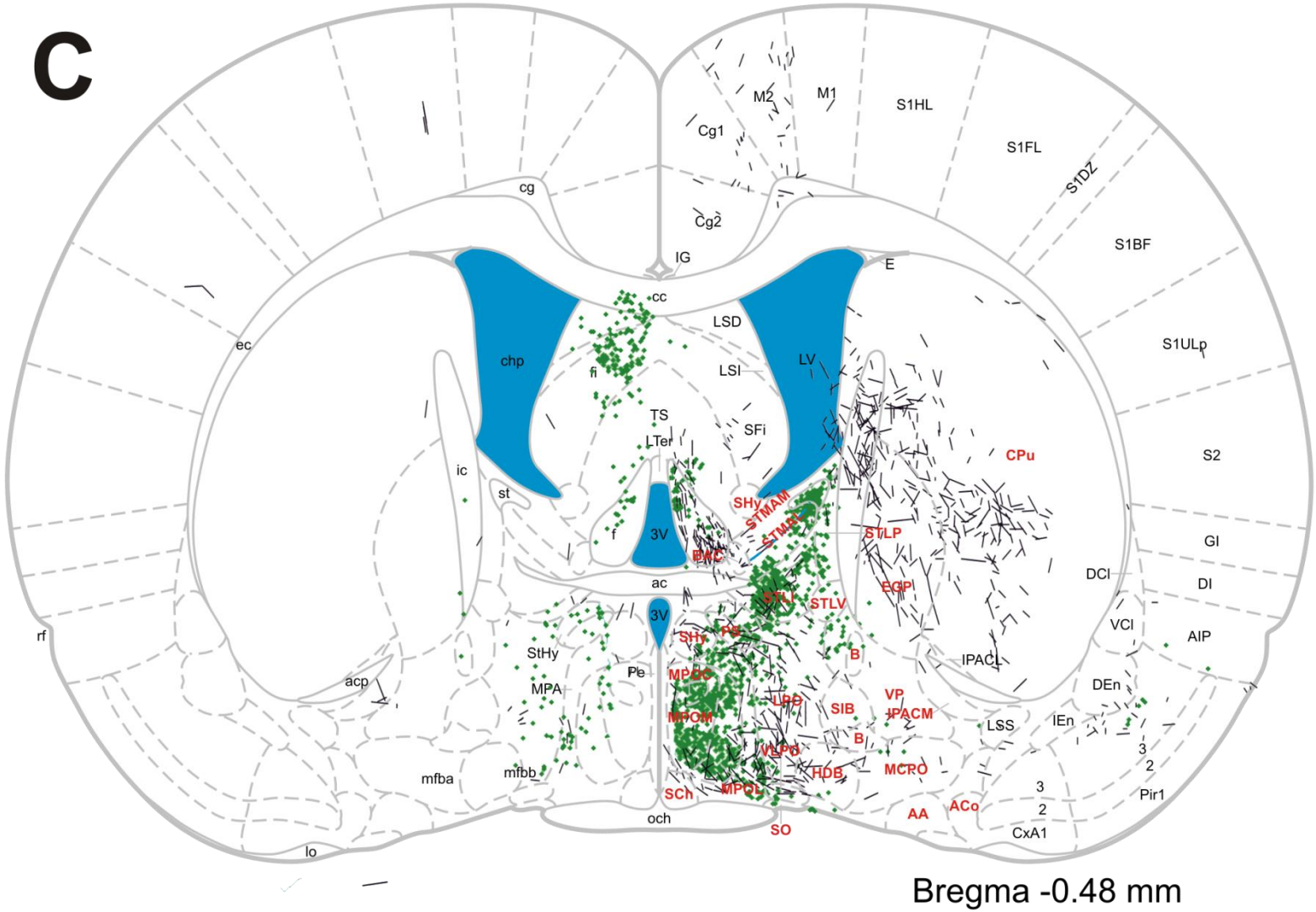


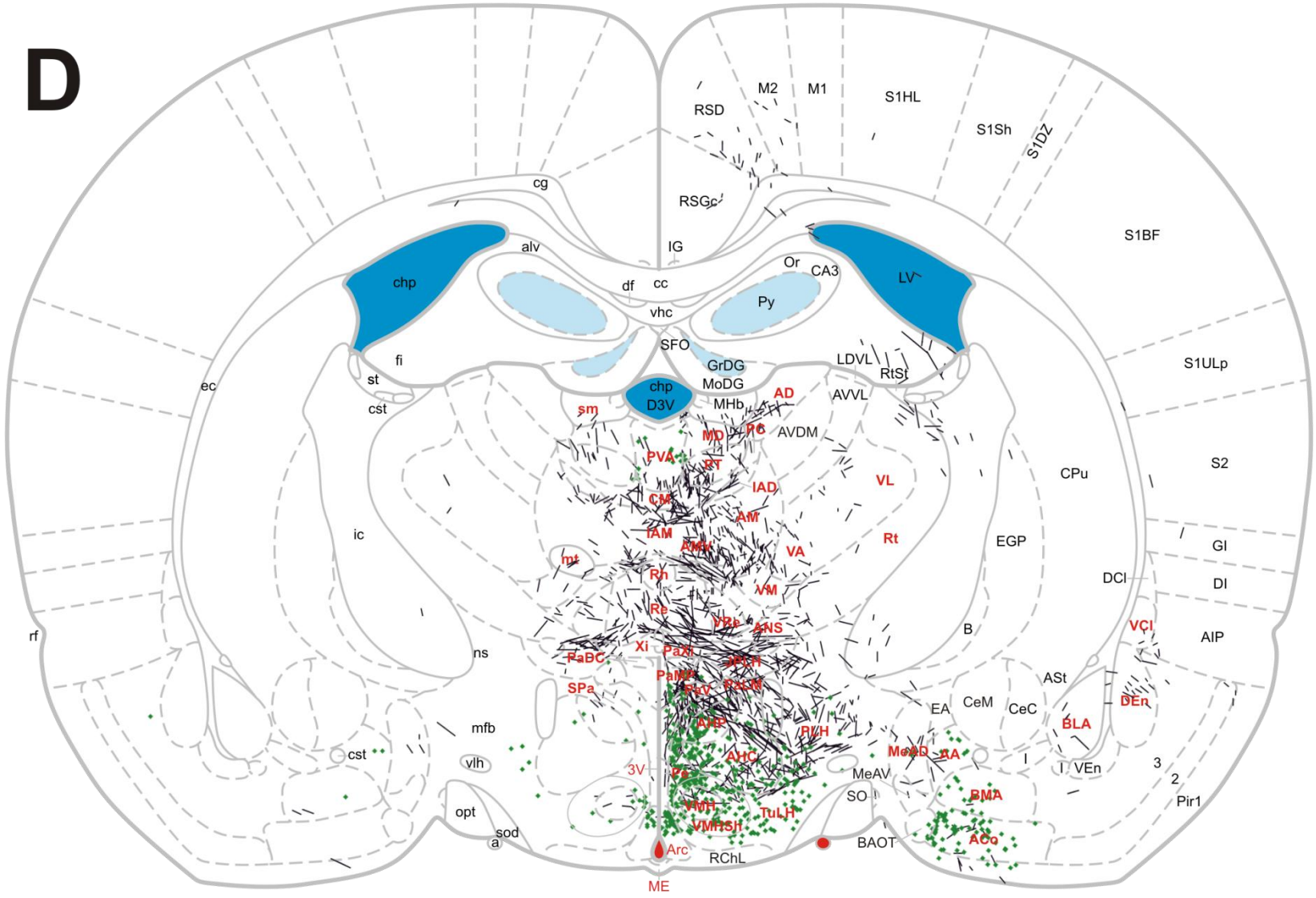
Figure 3 (A-K) – Schematic representation of retrogradely labeled cells (green dots) and anterogradelly labeled fibres (black lines) on representative coronal sections of the rat brain atlas. A – Represents coronal section at +3.0 mm Bregma; B – Represents coronal at + 0.36 mm Bregma line; C – Represents coronal section at -0.48 mm Bregma. D – Represents coronal section at -1.80mm Bregma. E - Represents coronal section at -3.24 mm Bregma. F - Represents coronal section at -4.56 mm Bregma. G - Represents coronal section at -5.64 mm Bregma. H – Represents coronal section at -8.52mm Bregma. I - Represents coronal section at -10.92 mm Bregma. J - Represent coronal section at -12.96 mm Bregma. K - Represent coronal section at -14.28 mm Bregma. For abbreviations see table 1.



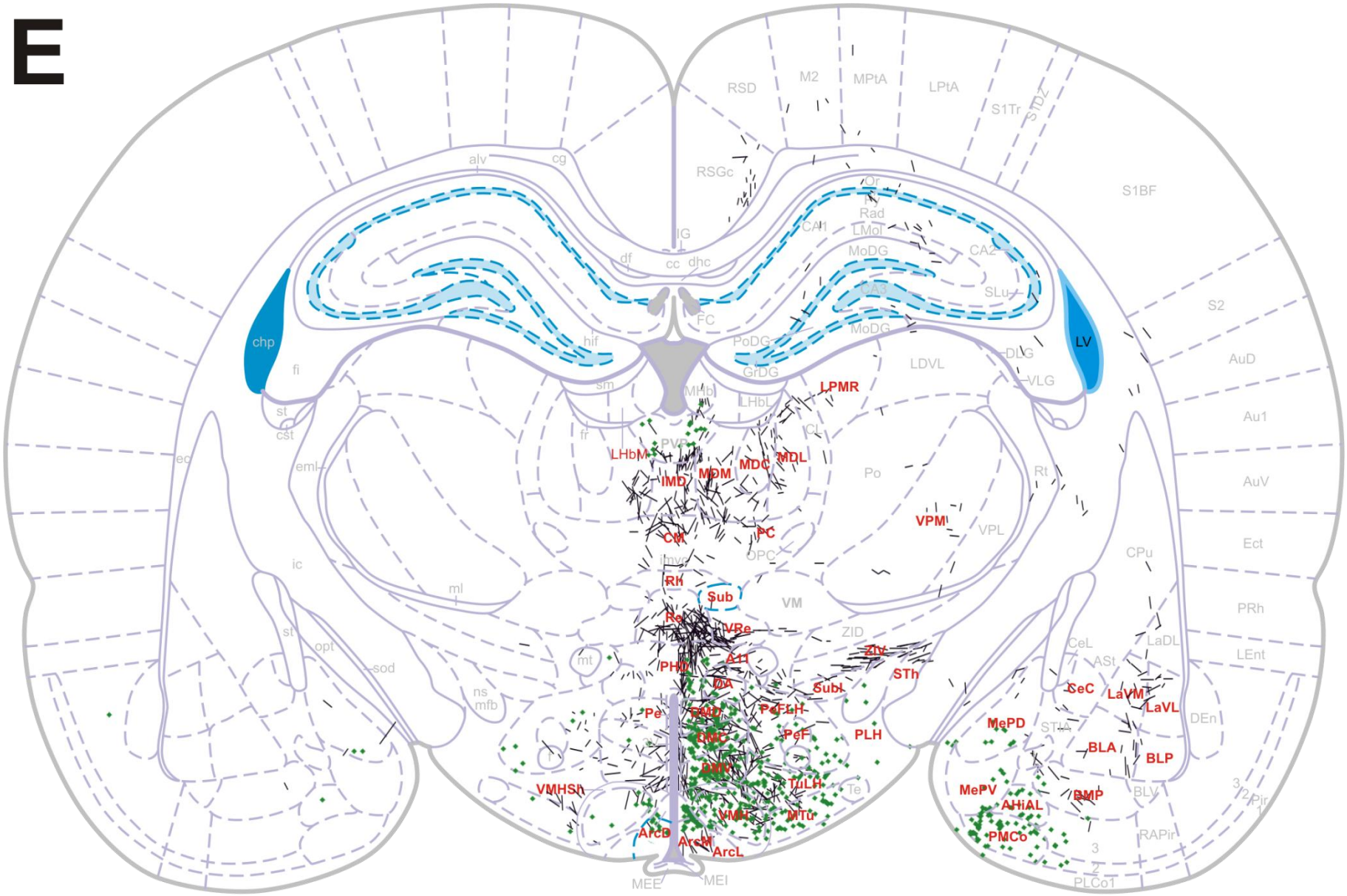
Bregma 0.36 mm



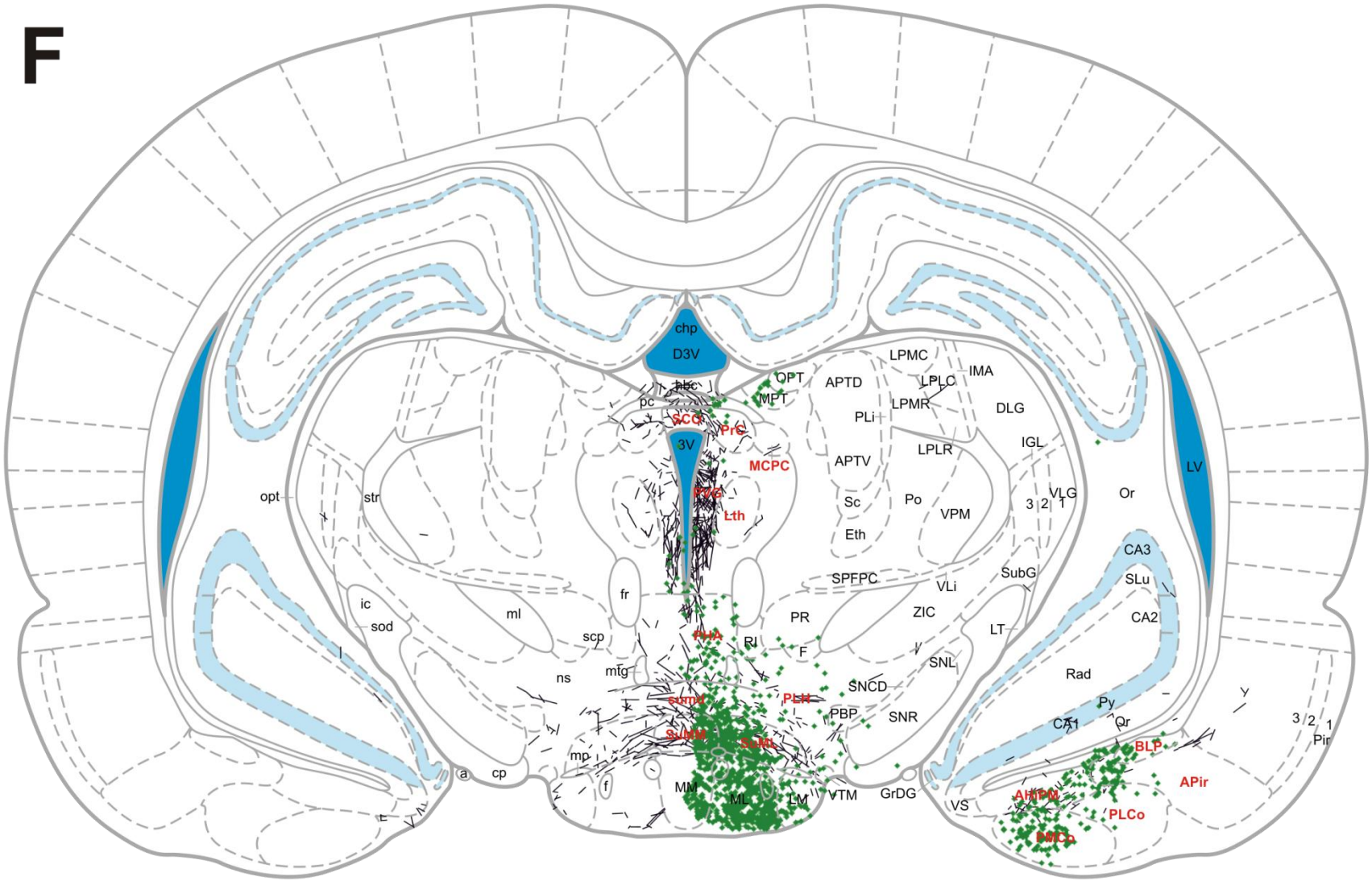
Bregma -0.48 mm



Bregma -1.80 mm

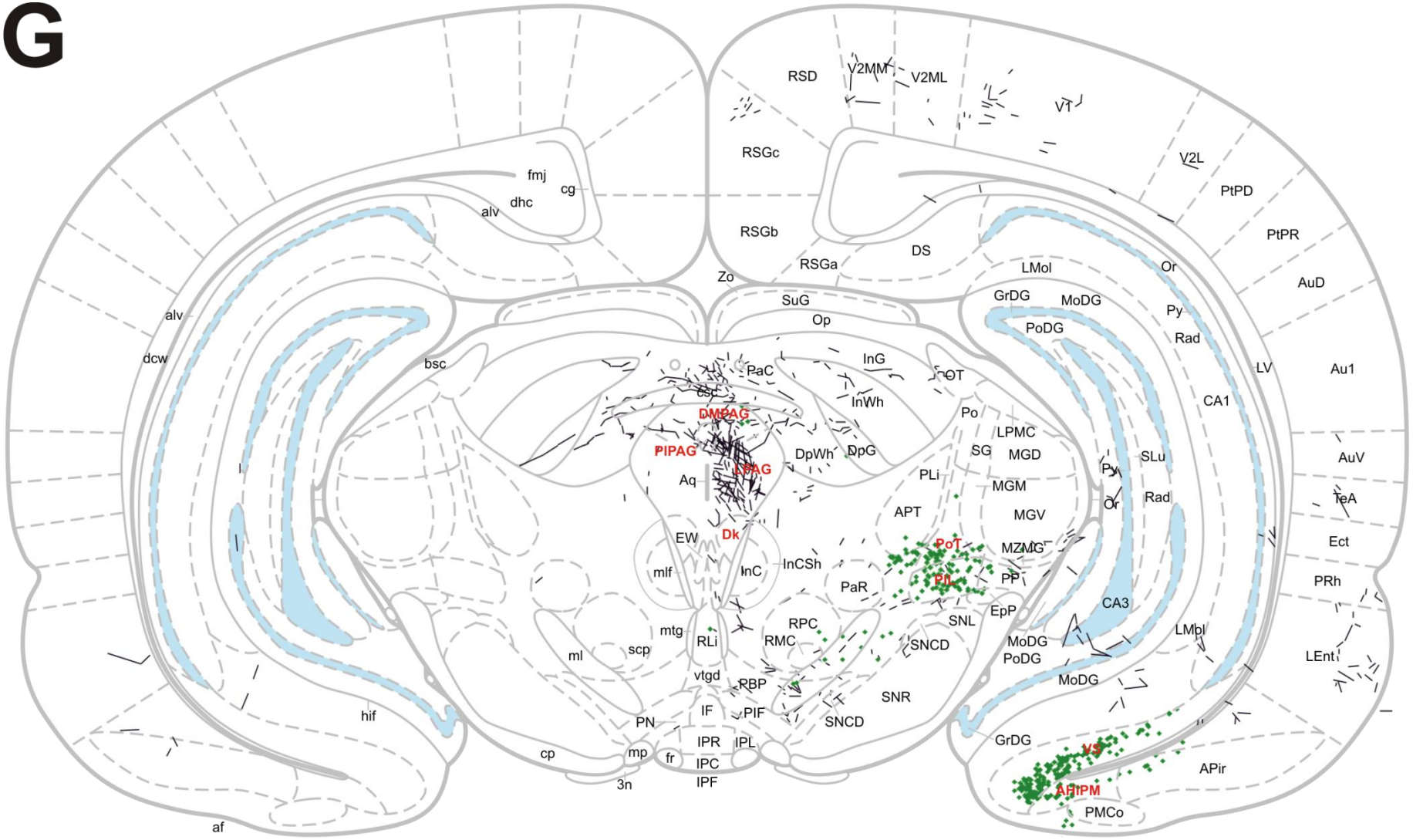


Bregma -3.24 mm



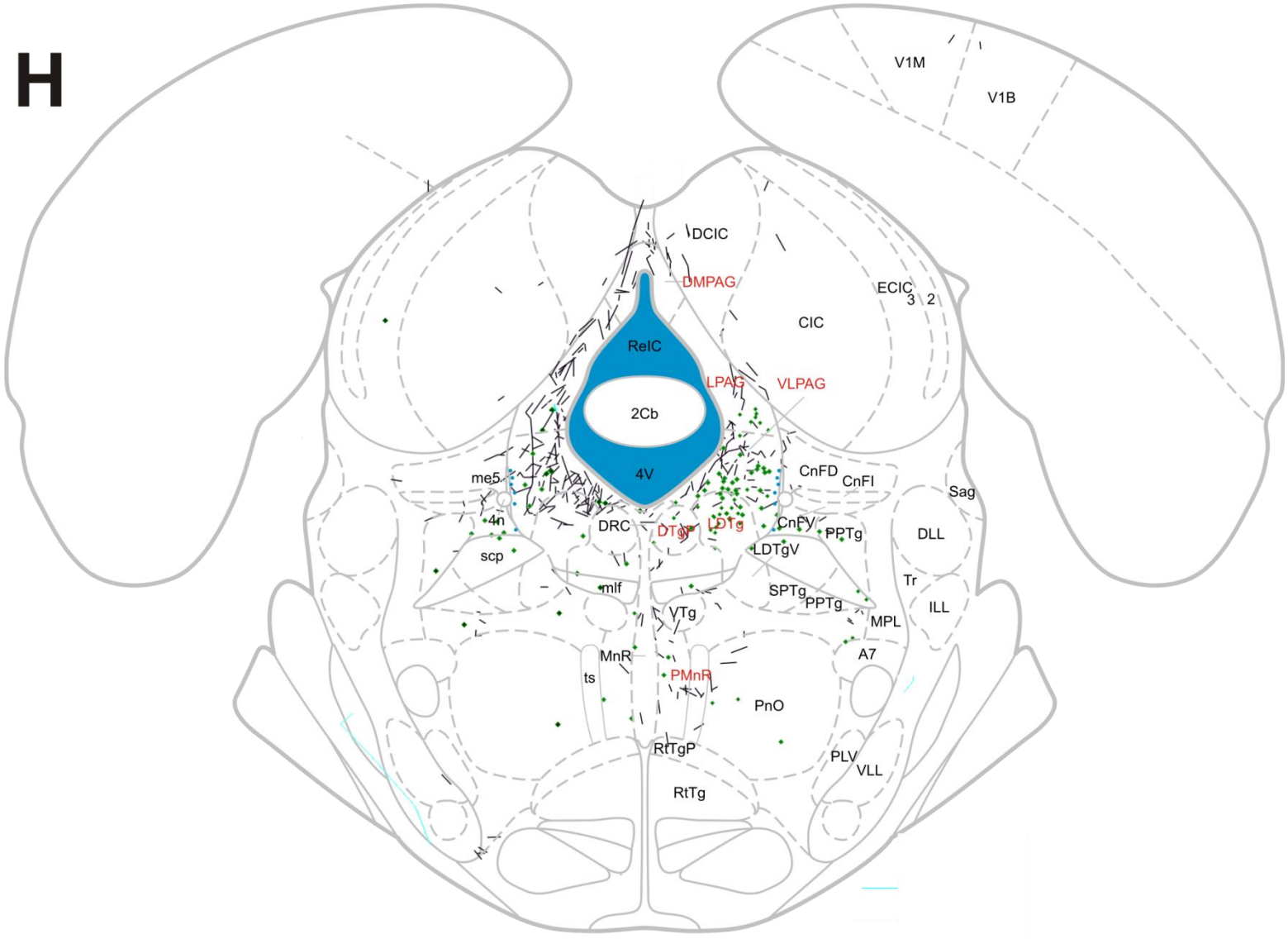
Bregma -4.56 mm

G



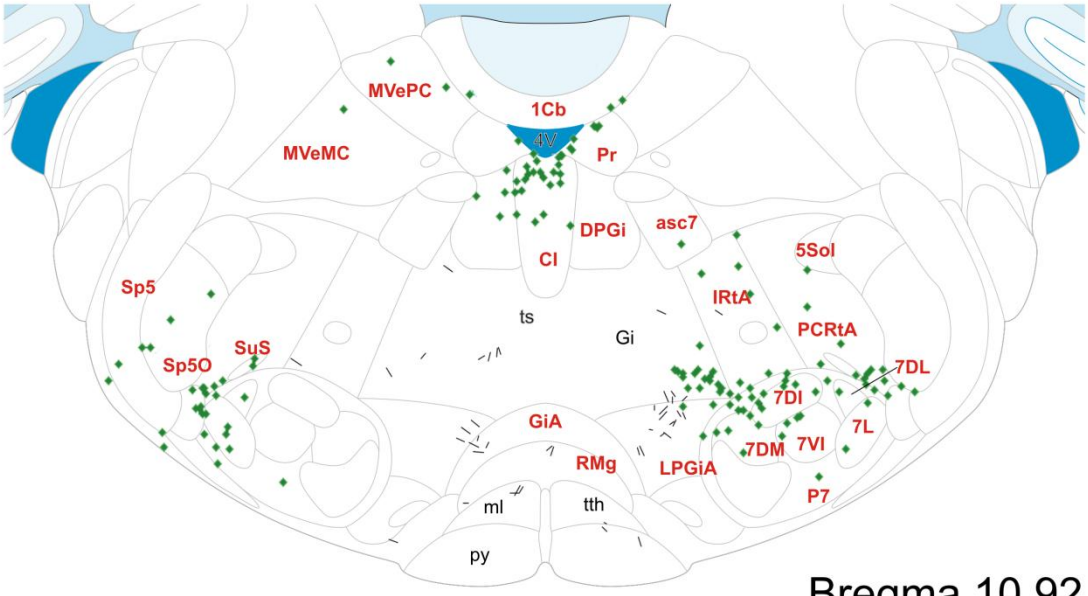
Bregma -5.64 mm

H



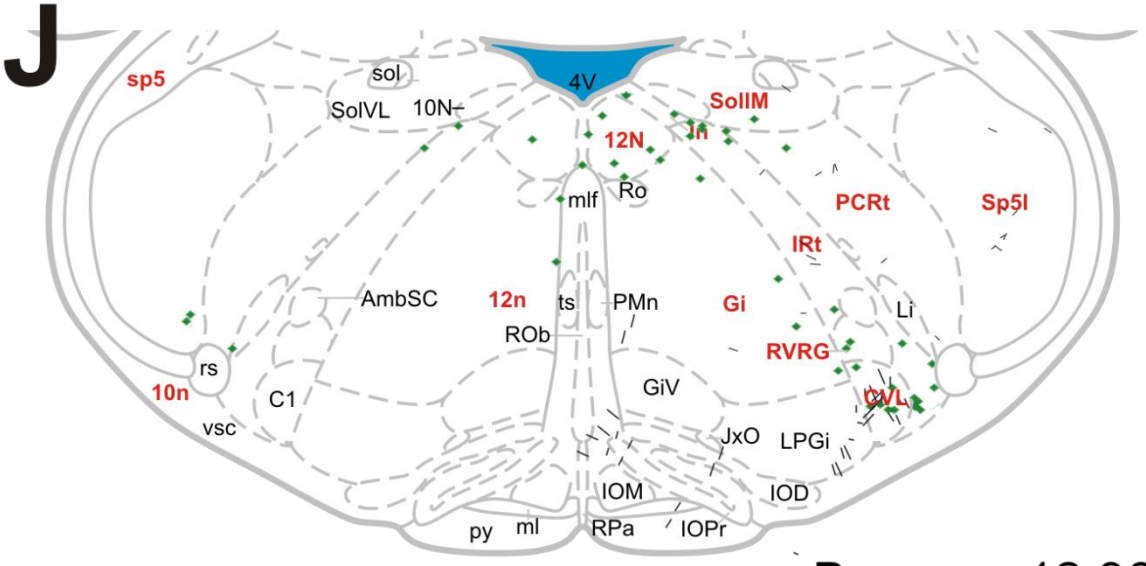
Bregma -8.52 mm

I

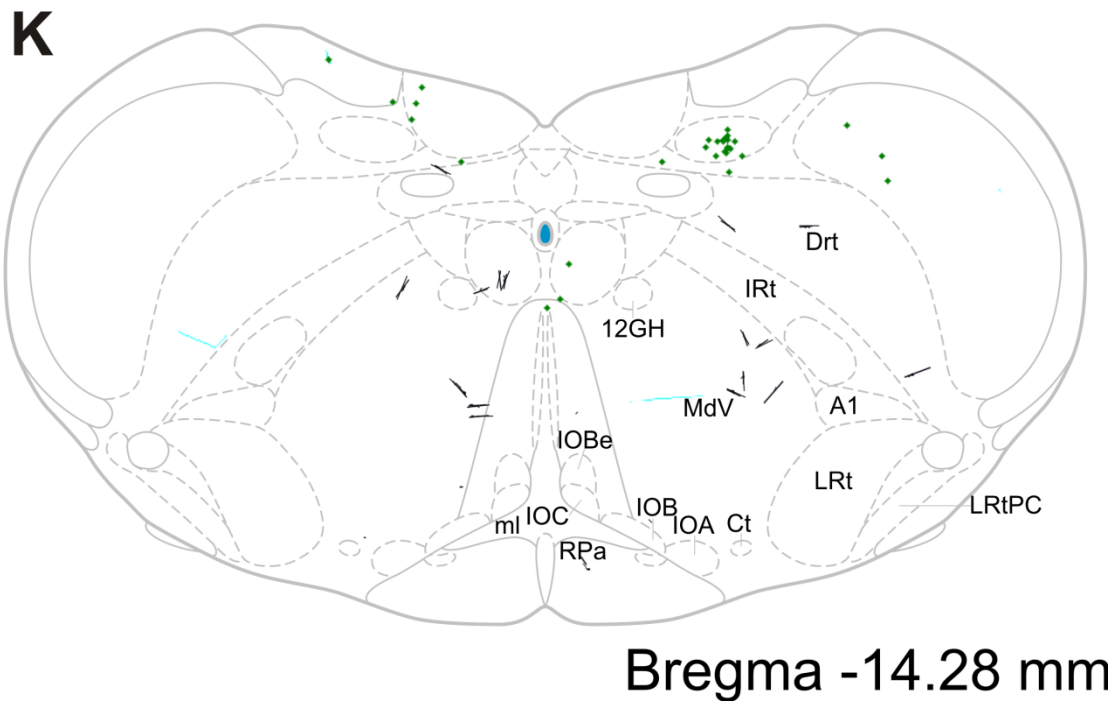


Bregma 10.92 mm

J



Bregma -12.96 mm


K
Drt
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Bregma -14.28 mm

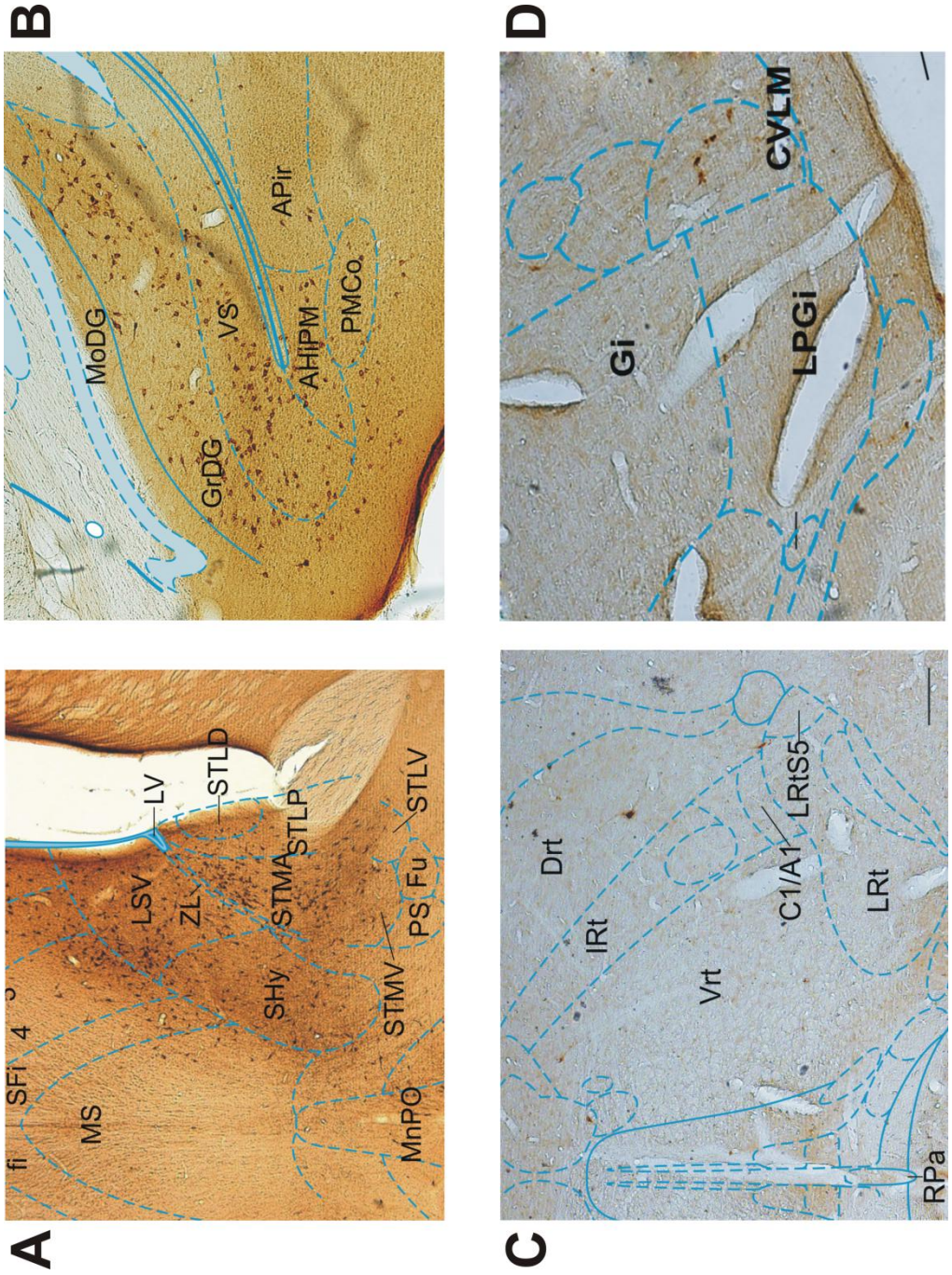

Figure 4 - Photomicrographs of brain coronal sections with retrogradely labeled neurones. A – Labeled neurones present in the bed nucleus of the stria terminalis (STMA) (RC: +0.12 mm Bregma). B – Stained neurones in the subiculum (VS) (RC: -5.52 mm Bregma). C – Modest staining of the medullary dorsal reticular nucleus (DRt) (RC: -13.92 mm Bregma). D. Stained neurones in the caudal ventrolateral medulla (CVLM) (RC: -12.96 mm Bregma).

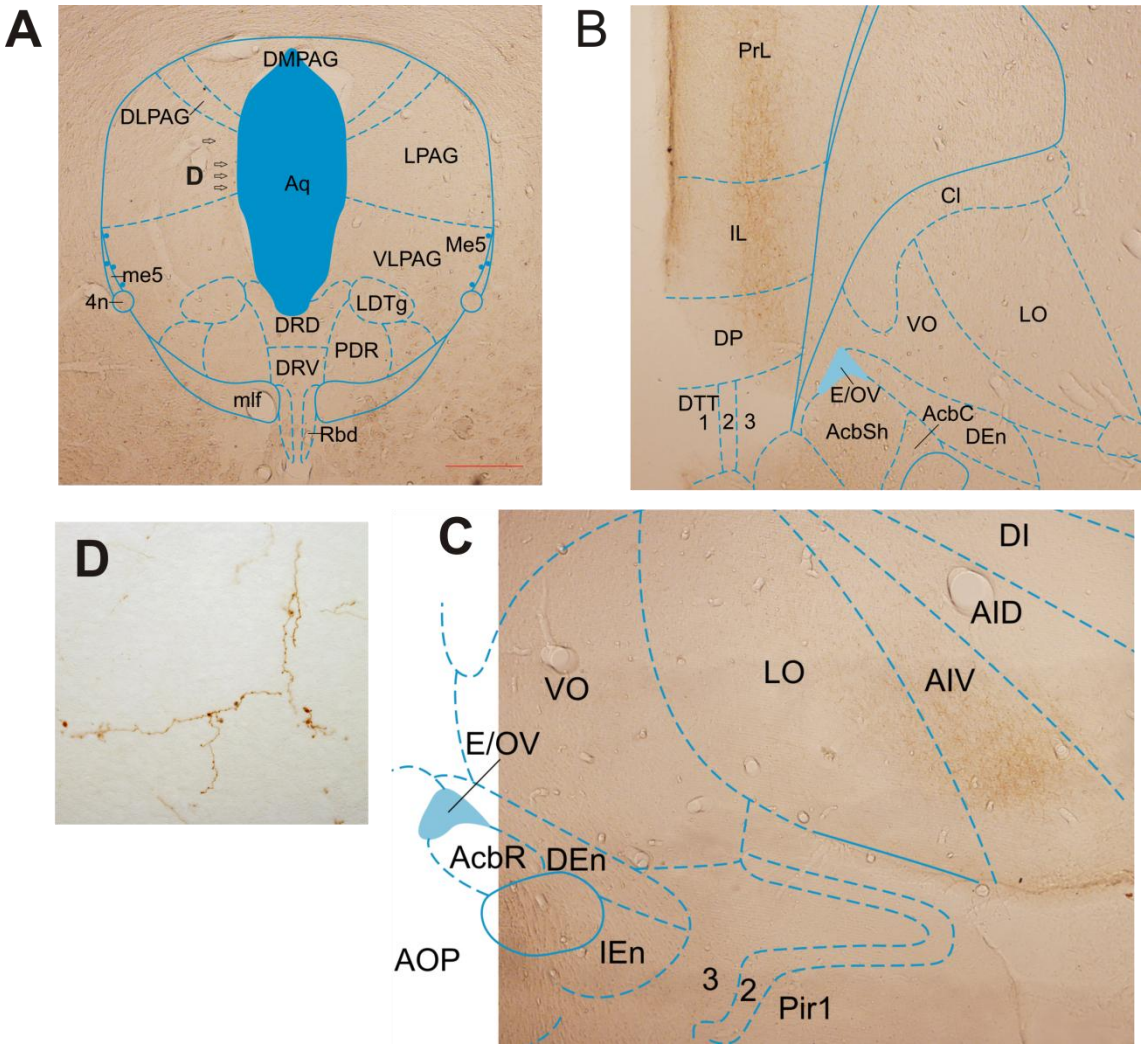

Figure 5 - Photomicrographs of brain coronal sections with anterogradely labeled fibres. A – Labeled fibres in the periaqueductal gray matter (PAG) (RC: -8.16 mm Bregma). B – Stained fibres in the prefrontal cortex (PrL) (RC: +3.00 mm Bregma). C - Stained fibres in the prefrontal cortex (PrL) (RC: +3.24 mm Bregma). D – Detail of labeled fibre corresponding to the area with arrows in image A.

Table 1 - Density of labeled neurones and fibres from the forebrain to the medulla of the rat after injection of retrograde and anterograde anatomical tracers in the paraventricular nucleus of the hypothalamus [*DRt corresponds to MdM and VRt to MdV in Paxinos and Watson (2005) atlas].

		Retrograde		Anterograde	
Telencephalon		IP	CL	IP	CL
AA	Anterior amygdaloid area			+	
aca	Anterior commissure, anterior part	18	1		
AcbC	Nucleus accumbens, core			+++	+
AcbR	Nucleus accumbens, rostral pole			+	
AcbSh	Nucleus accumbens, the shell of the	26	3	++	+
ACo	anterior cortical amygdaloid nucleus			+	
AHiPM	Amygdaloihippocampal area, posteriomedial	21		+++	
AID	Agranular insular cortex, dorsal part			+++	
AIP	Agranular insular cortex, posterior part			++	
AIV	Agranular insular cortex, ventral part			+++++	
AOM	Anterior olfactory nucleus, anterior part	1			
AOP	Anterior olfactory nucleus, posterior part	7		+	
APir	Amygdalopiriform transition area	17		+	+
B	Basal nucleus			+	
BAOT	Bed nucleus of the accessory olfactory tract	1			
BLA	Basolateral amygdaloid nucleus, anterior part	10	1	+++	
BLP	Basolateral amygdaloid nucleus, posterior	3		+++	
BLV	Basolateral amygdaloid nucleus, ventral			+	
BMA	Basomedial amygdaloid nucleus, anterior part	4		+	
BMP	Basomedial amygdalois nucleus, post. part	1		+	
BSTIA	BN stria terminalis, intraamygdaloid	18			
BSTL	Bed nucleus of the stria terminalis, lateral	114	8		
BSTLi	BN stria terminalis, medial intermediate	189	24		
BSTMA	BN of the stria terminalis, medial anterior	422	35		
BSTMPI	BN stria terminalis, medial posterointerm.	99	2		
BSTMPM	BN stria terminalis, medial posteromedial	21			
BSTMV	BN stria terminalis, medial ventral	60			
C1	Adrenaline cells	90	15	++	
CB	Cell bridges of the ventral striatum			++	
CeL	Central amygdaloid nucleus, lateral division	2			
CeM	Central amygdaloid nucleus, medial division	4		++	
Cg1	Cingulated cortex, arca 1	1	1	++	+
Cg2	Cingulated cortex, arca 2	1		+	+
CPu	Caudate putamen (striatum)	4		++++	+
DCl	Dorsal part of claustrum			+	
Den	Dorsal endopiriform nucleus	24	5	++++	
DI	Dysgranular insular cortex			++	
DP	Dorsal peduncular cortex	36	8	+	
DTT	Dorsal tenia tecta	68	17		
EAC	Sublenticular extended amygdala, central			++	
EAM	Sublenticular extended amygdala, medial			+	+
HDB	nucleus horizontal limb of the diagonal band			++	++
ICj	Islands of calleja	7			
ICjM	Islands of calleja, major islands	5			
IEn	Intermediate endopiriform nucleus			+	
IL	Infralimbic cortex	58	6	++	

		Retrograde		Anterograde	
Telencephalon (Cont.)		IP	CL	IP	CL
LAcbSh	Lateral accumbens shell			++	
LaVL	Lateral amygdaloid nucleus, ventrolateral part	6		+	
Ld	Lambdoid septal zone	8		+	+
LEnt	Lateral entorhinal cortex			++	+
LSI	Lateral septal nucleus, intermediate part	247	20	+	++
LSS	Lateral stripe of the striatum	3			
LSV	Lateral septal nucleus, ventral part	500	13		
Lo	Lateral orbital cortex			++++	
M1	Primary Motor Cortex			+	+
M2	Secondary Motor Cortex			++	+
MeAD	Medial amygdaloid nucleus, anteriordorsal			+	
MePD	Medial amygdaloid nucleus, posterodorsal	36		+	
MePV	Medial amygdaloid nucleus, posteroventral	10			
Mfba	Medial forebrain bundle, a component		165		
Mfbb	Medial forebrain bundle, b component	119			
MS	Medial septal nucleus	25	30	+	
Nv	Navicular nucleus of the basal forebrain			+	
PMCo	Posterior cortical amygdaloid nucleus (C3)	47			
PMLo	Posterolateral cortical amygdaloid nucleus	6			
PaC	Paracommissural nucleus, post. commissure			+	
PRh	Perirhinal cortex			+	
PrL	Prelimbic cortex	17	6	++	
RSD	Retrosplenial dysgranular cortex			+	
RSGc	Retrosplenial granular cortex, c region			+	
S2	Secondary somatosensory cortex			+	
SHi	Septohippocampal nucleus			++	+
SIB	Substancia innominata, dorsal part	8	5	++	++
STMPM	BNST, medial division, posteromedial part			++++	
STMAM	BNST, medial division, anteromedial part			+	+
STMAL	BNST, medial division, anterolateral part			+	
STLI	BNST, lateral division, intermediate part			++	
V2MM	Secondary visual cortex, mediomedial area			++	
V2ML	Secondary visual cortex, mediolateral area			+	
V1	Primary visual cortex			+++	
V2L	Secondary visual cortex, lateral area			+	
VCl	Ventral part of claustrum			++	
VEn	Ventral endopiriform nucleus			++	
VO	Ventral orbital cortex			++	
VP	Ventral palladium	34		++++	++
VS	Ventral subiculum	114			
TeA	Temporal associatin cortex			+	
TS	Triangular septal nucleus			+	
Tu1	Olfactory tubercle			++	+

		Retrograde		Anterograde	
Diencephalon		IP	CL	IP	CL
A11	Dopaminergic cells	3			
A13	A13 dopamine cells			++	+
AD	Anterodorsal thalamic nucleus			+	
ADP	Preoptic nucleus, anterodorsal part	21			
AHA	Anterior hypothalamic area, anterior part			++++	
AHC	Anterior hypothalamic area	18			
AHP	Posterior hypothalamic area	7	2		
AM	Anteromedial thalamic nucleus			+	
AMV	Anteromedial thalamic nucleus, ventral part			++	
ANS	Accessory neurosecretory nuclei			+++	++
ArcD	Arcuate nucleus, dorsal part	28			
ArcLP	Arcuate hypothalamic nucleus, lat. posterior	15	2		
ArcM	Arcuate nucleus, medial part	25	10	+	
ArcMP	Arcuate hypothalamic nucleus, medial post.	25	9		
AuV	Secondary auditory cortex, ventral area			+	
AVD	Anterovent thalamic nucleus, dorsal			+++	
AVPe	Anteroventral periventricular nucleus	26	8		
CM	Central medial thalamic nucleus			++	+
DA	dorsal hypothalamic area			++++	++
DMC	Dorsomedial hypothalamic nucleus, compact	8	2	++++	+
DMD	Dorsomedial hypothalamic nucleus, dorsal	42	3	++++	+
DMV	Dorsomedial hypothalamic nucleus, ventral	10	5	++++	
DTM	Tuberomammillary nucleus, dorsal part	6			
IAD	Interanterodorsal thalamic nucleus			++	+
IMD	Intermediodorsal thalamic nucleus			++	++
JPLH	Juxtaparaventricular lateral hypothalamus			++++	++
LA	Lateroanterior hypothalamic nucleus			+++	
LDVL	Laterodorsal thalamic nucleus, ventrolateral			++	
LHbL	Lateral habenular nucleus, lateral part			++	
LPO	Lateral preoptic area	23	15	++	+
mch	Medial corticohypothalamic tract	12	3		
MDC	Mediodorsal thalamic nucleus, central part			+++	+
MDL	Mediodorsal thalamic nucleus, lateral part			++	++
MDM	Mediodorsal thalamic nucleus, medial part	3		++	++
MHb	Medial habenular nucleus			++	
MPA	Medial preoptic area	240	80	+	+
MPOC	Medial preoptic nucleus, central part	8	2	+	
MPOL	Medial preoptic nucleus, lateral part		8	++	
MPOM	Medial preoptic nucleus, medial part	56	14	+++	+
MRe	Mammillary recess of the 3rd ventricule	5			
MTu	Medial tuberal nucleus	3		++	
PaAP	Paraventricular hypothalamic nucleus, ant. AP	91	13		

		Retrograde		Anterograde	
Diencephalon (Cont.)		IP	CL	IP	CL
PamP	Paraventricular hypothalamic nucleus, medial	4		++	
PaV	Paraventricular hypothalamic nucleus, ventral	2			
PC	Paracentral thalamic nucleus			++	+
PDP	Periventricular hypothalamic nucleus	6	1		
Pe	Periventricular nucleus	67	6		
PeF	Perifornical nucleus			++	
PeFLH	Perifornical part of lateral hypothalamus			++	+
PH	Posterior hypothalamic area	50	6		
PHD	Posterior hypothalamic area, dorsal part			++++	
PIL	Posterior intralaminar thalamic nucleus	14	11		
PLH	Peduncular part of lateral hypothalamus			+++	
POT	Posterior thalamic nuclear group, triangular	13	7		
PS	Parastrial nucleus	36		+++	
PT	Paratenial thalamic nucleus			+	+
Pv	Periventricular fiber system	4		++	
PVA	Paraventricular thalamic nucleus, anterior			+	
PVP	Paraventricular thalamic nucleus, posterior	6		++	++
RCh	Retrochiasmatic area	52	8	+	+
Re	Reuniens thalamic nucleus			++	+
Rh	Rhomboid thalamic nucleus			+	
Rt	Reticular thalamic nucleus			+	+
S1FL	Primary somatosensory cortex, forelimb reg.			+	
S1HL	Primary somatosensory cortex, hindlimb reg.				+
Sch	Suprachiasmatic nucleus	17	3		
SFi	Septofimbrial nucleus			+	
SM	Nucleus of the stria medullaris			++	
SO	Supraoptic nucleus		2		
SHy	Septohypothalamic nucleus			++	
StHy	Striohypothalamic nucleus	20			
SubD	Submedius thalamic nucleus, dorsal part			++++	++
SubV	submedius thalamic nucleus, ventral part			++	+
TC	Tuberum-cinerum	17			
TuLH	Tuberal region of lateral hypothalamus			+++	+
VLH	Ventrolateral hypothalamic nucleus			++	
VLPo	Ventrolateral preoptic nucleus	2		+	
VMHC	Ventromedial hypothalamic nucleus, central			+++	
VMHDM	Ventromedial hypot. nucleus, dorsomedial	41	2	++++	+
VMHSh	ventromedial nucleus hypothalamus shell			++	
VMHVL	Ventromedial hypoth. nucleus, ventrolat.	44	1	++	
VMPo	Ventromedial preoptic nucleus	43	10		
VPL	ventral posterolateral thalamic nucleus			++	+
VPM	ventral posteromedial thalamic nucleus			++	+
VPPC	Ventral posterior thalamic nucleus, parv.	8			
VRe	Ventral reunions thalamic nucleus	4		+	+
VTM	Tuberomammillary nucleus, ventral part	2			
ZID	Zona incerta, dorsal part			+++	
ZIV	Zona incerta, ventral part	2		+++	

		Retrograde		Anterograde	
Mesencephalon		IP	CL	IP	CL
com	commissural nucleus of the inferior colliculus			+	
CnFI	Cuneiform nucleus, intermediate part			+	+
CnFV	Cuneiform nucleus, ventral part			+	+
DLPAG	Dorsolateral periaqueductal gray	24	18	++	+
DMPAG	Dorsomedial periaqueductal gray	9	2	++	
DpMe	Deep mesencephalic nucleus	49	7	+	
DpG	Deep gray layer of the superior colliculus			+	+
DpWH	Deep white layer of the superior colliculus			+	+
DRC	Dorsal raphe nucleus, caudal part			+	
DrD	Dorsal raphe nucleus, dorsal part			+	
DrL	Dorsal raphe nucleus, lateral part			++	
DTgP	Dorsal tegmental nucleus, pericentral part			++	+
InG	Intermediate gray layer superior colliculus			+	
InWh	intermediate white layer superior colliculus			+	+
IPC	Interpeduncular nucleus, caudal part	1	3		
IPDL	Interpeduncular nucleus, dorsolateral part	1	2	+	
IPI	Interpeduncular nucleus, intermediate subnucleus	6			
IPR	Interpeduncular nucleus, rostral subnucleus	3			
	Nigrostatial bundle	10	7		
LDTg	Laterodorsal tegmental nucleus			++	+
LDTgV	Laterodorsal tegmental nucleus, ventral			+	
LPAG	Lateral periaqueductal gray			+++	+
ML	Medial mammillary nucleus, lateral part	12	12		
MM	Medial mammillary nucleus, medial part	17	10		
MnR	Median raphe nucleus			+	+
MZMG	Marginal zone of the medial geniculate	1		+	
OT	Nucleus of the optic tract			+	
PaC	Paracommissural nucleus post. commissure			++	+
PaR	Pararubral nucleus			+	+
PIL	Posterior intralaminar thalamic nucleus	40		+	
PIPAG	Plioglial part of periaqueductai gray			+	+
PMnR	Paramedian raphe nucleus			+	+
PN	Paranigral nucleus of the VTA			+	
PnO	Pontine reticular nucleus, oral part			+	
PoT	Posterior thalamic nuclear group, triangular	22		+	
PP	Peripeduncular nucleus	1		+	
PPtg	Pedunculopontine tegmental nucleus			+	+
RBP	Rhabdoid nucleus			+	
RLi	Rostral linear nucleus of the raphe	1		+	
RPC	Red nucleus, parvicellular part	2			
scp	Superior cerebellar peduncule	10			
SNR	Substantia nigra, reticular part	1			
SNCD	Substantia nigra, compact part, dorsal tier			+	
VLPAG	Ventrolateral periaqueductal gray			++++	+++
VTA	Ventral tegmental area		1		

		Retrograde		Anterograde	
Pons and medulla		IP	CL	IP	CL
7L	Facial nucleus, lateral subnucleus	2	3		
7DI	Facial nucleus, dorsal interm. subnucleus	8			
7DL	Facial nucleus, dorsolateral subnucleus	1	1		
7DM	Facial nucleus, dorsomedial subnucleus	5			
7VI	Facial nucleus, ventral intermediate subnucleus	5			
asc7	ascending fibers of the facial nerve	1			
CI	Caudal interstitial nucleus of the medial longitudinal fasciculus	7	11		
DPGi	Dorsal paragigantocellular nucleus		6		
Gi	Gigantocellular reticular nucleus	15		+	++
GiA	Gigantocellular reticular nucleus, alpha part				+
IRtA	Intermediate reticular nucleus, alpha part	5		+	
LPGiA	Lateral paragigantocellular nucleus. alpha	2		++	+
DRt*	Dorsal reticular nucleus			+	
VRt*	Ventral reticular nucleus			+	
ml	Medial lemniscus				+
ML	Medial mammillary nucleus, lateral part				
MVePC	medial vestibular nucleus, parvicellular part		2		
MVeMC	medial vestibular nucleus, magnocellular		1		
P7	Perifacial zone	12	5		
PCRtA	Parvicellular reticular nucleus, alpha part	9			
Pr	Prerubral field	6	2		
RMg	Raphe magnus nucleus			+	
RPa	Raphe pallidus nucleus			+	
Sp5	Spinal trigeminal tract		2		
Sp5O	Spinal trigeminal nucleus, oral part	2	3		
SuS	Superior salivatory nucleus	1	2		

Chapter 2.3

Hypothalamic descending pain modulation

Chapter 2.3.1

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Research Report

Influence of arthritis on descending modulation of nociception from the paraventricular nucleus of the hypothalamus

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ABSTRACT

We studied the influence of arthritis on descending modulation of nociception from the hypothalamic paraventricular nucleus (PVN) in the rat. Spinal nociception was assessed by the heat-evoked limb withdrawal in awake animals while neuronal responses were recorded in a potential brainstem relay, the rostroventromedial medulla (RVM), under pentobarbitone anesthesia. Following injection into the PVN, glutamate attenuated and lidocaine enhanced nociceptive spinal reflex responses in arthritic and control animals. In controls, PVN-induced antinociception was reversed by spinal administration of a 5-HT_{1A} receptor or an α_2-adrenoceptor antagonist but not by an opioid receptor antagonist. In arthritic animals, PVN-induced antinociception was not reversed by a 5-HT_{1A} receptor antagonist, while the roles of α_2-adrenoceptors or opioid receptors could not be assessed due to significant actions of antagonists alone. The spontaneous activity of presumably pronociceptive ON-cells of the RVM and that of antinociceptive OFF-cells was increased in arthritis. Lidocaine in the PVN increased ON-cell firing in control animals and decreased OFF-cell firing in arthritic animals, while glutamate failed to affect activity of RVM cells. The results indicate that the PVN influences phasic and tonic descending antinociception in arthritic as well as control conditions, and the RVM may contribute to the relay of this influence. In arthritis, the neurochemistry of descending antinociception differs at least partly from that in controls. Arthritis has a dual influence on the PVN-induced drive of relay cells in the RVM which reduces the arthritis-induced net change in the descending antinociceptive influence from the PVN.

1. Introduction

The paraventricular nucleus (PVN) of the hypothalamus is involved in descending modulation of nociception. This is indicated by the finding that electrical or chemical stimulation of the PVN has produced spinal antinociception (Condés-Lara et al., 2006; Miranda-Cardenas et al., 2006; Shiraishi et al., 1995; Wang et al., 1990a; Yang et al., 2006; Yirmiya et al., 1990). In line with this, lesions of the PVN facilitated nociception (Yang et al., 2006) and attenuated stress-induced analgesia (Truesdell and Bodnar, 1987), although not in all experimental conditions (Fuchs and Melzack, 1996; Lariviere et al., 1995). Efferent connections to the spinal dorsal horn directly or indirectly through various relay nuclei in the brainstem, such as the periaqueductal gray and the raphe magnus, provide a potential anatomical substrate for the descending antinociceptive action

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Abbreviations: PVN, paraventricular nucleus of the hypothalamus; RVM, rostroventromedial medulla; WDR, wide-dynamic range

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induced by the PVN (Holstege, 1987; Swanson and Sawchenko, 1983).

The role of various descending pathways and the neurochemistry underlying PVN-induced antinociception is only partly known. Early studies suggested that spinal antinociception induced by PVN stimulation is not dependent on opioid receptors or vasopressin (Shiraishi et al., 1995; Yirmiya et al., 1990). More recent studies, however, suggest that opioid receptors have a minor contribution to the PVN-induced antinociception (Yang et al., 2006; Miranda-Cardenas et al., 2006), while vasopressin (Yang et al., 2006) or oxytocin (Condés-Lara et al., 2006; Miranda-Cardenas et al., 2006) play a major role in mediating the descending antinociceptive action from the PVN. This is in line with a substantial number of hypothalamo-spinal cells that are stained with antisera directed against vasopressin or oxytocin (Cechetto and Saper, 1988; Condés-Lara et al., 2007; Swanson and Sawchenko, 1983). Recent results indicate that activation of GABAergic spinal interneurons by oxytocin may be involved in mediating the PVN-induced antinociception at the spinal cord level (Rojas-Piloni et al., 2007). Although the PVN-induced descending antinociception may be explained by direct hypothalamo-spinal connections, the potential role of various brainstem nuclei in mediating the antinociceptive action from the PVN to the spinal cord still remains to be studied. Concerning potential brainstem relay nuclei and neurotransmitters mediating their action, it is not yet known whether the PVN-induced spinal antinociceptive action involves monoaminergic neurotransmitters, such as serotonin (5-HT) or norepinephrine, that are known to have an important role in descending modulation of nociception (Pertovaara, 2006; Yaksh, 2006).

Pathophysiological conditions may induce significant changes in the function of descending pain-modulatory pathways leading to facilitation or attenuation of nociception (Pertovaara and Almeida, 2006; Vanegas and Schaible, 2004). In experimental arthritis, for example, the descending inhibition of afferent barrage from the inflamed joint was enhanced (Schaible et al., 1991). While it is known that arthritis is associated with changes in the expression of neuropeptides in the PVN (Shanks et al., 1998), it is not known whether the modulation of nociception descending from the PVN is changed in arthritis.

In the present investigation, we studied whether modulation of spinal nociception by the PVN is changed in arthritis. Also, we studied whether neurons in the rostroventromedial medulla (RVM), a final common pathway for many descending pathways (Gebhart, 2004), might have a role in mediating descending modulation of nociception from the PVN of control or arthritic animals. Furthermore, we assessed the roles of spinal noradrenergic α_2, serotoninergic 5-HT_{1A} and opioidergic receptors in mediating the descending modulation of nociception from the PVN by intrathecal microinjections of selective receptor antagonists in control and arthritic animals.

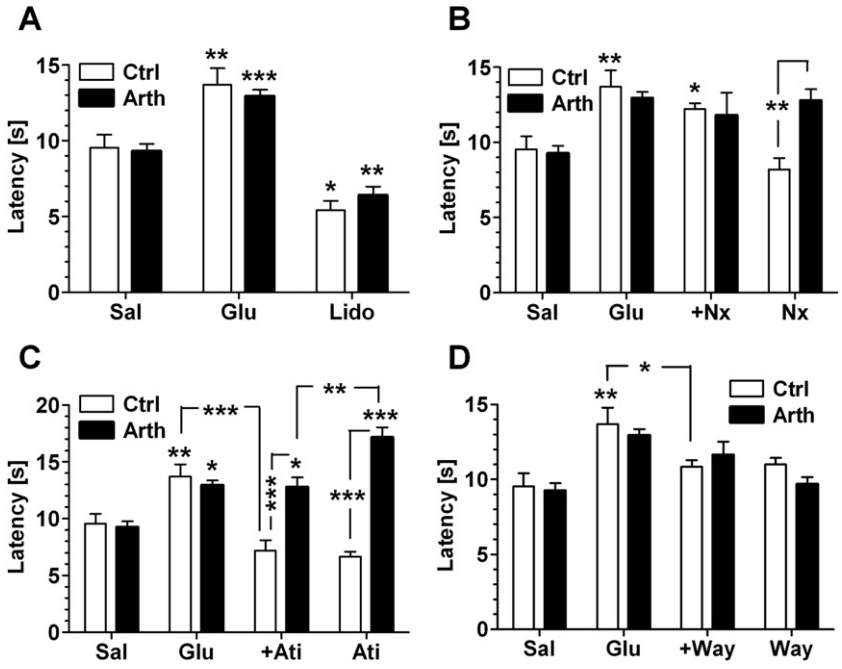

Fig. 1 – Mean latencies of heat-evoked limb withdrawal responses following administration of glutamate (Glu) or lidocaine (Lido) into the hypothalamic paraventricular nucleus (PVN) of control (Ctrl) or arthritic (Arth) animals. The noxious test stimulus was applied to the hind paw that was ipsilateral to the inflamed knee joint in arthritic animals. A) Influence of glutamate or lidocaine alone. B) Attempted reversal of glutamate-induced effect by spinal administration of an opioid receptor antagonist, naloxone (+Nx), and the effect of spinal administration of naloxone alone (Nx). C) Attempted reversal of glutamate-induced effect by spinal administration of an α_2-adrenoceptor antagonist, atipamezole (+Ati), and the effect by spinal administration of atipamezole alone (Ati). D) Attempted reversal of glutamate-induced effect by spinal administration of a 5-HT_{1A} receptor antagonist, WAY-100635 (+Way), and the effect by spinal administration of WAY-100635 alone (Way). The error bars represent S.E.M. (n=5–7). Unless specified otherwise, the asterisks indicate differences within groups (reference: the corresponding saline or Sal-group). *P<0.05, **P<0.01, ***P<0.005 (within groups: Dunnett's test; between groups: t-test).

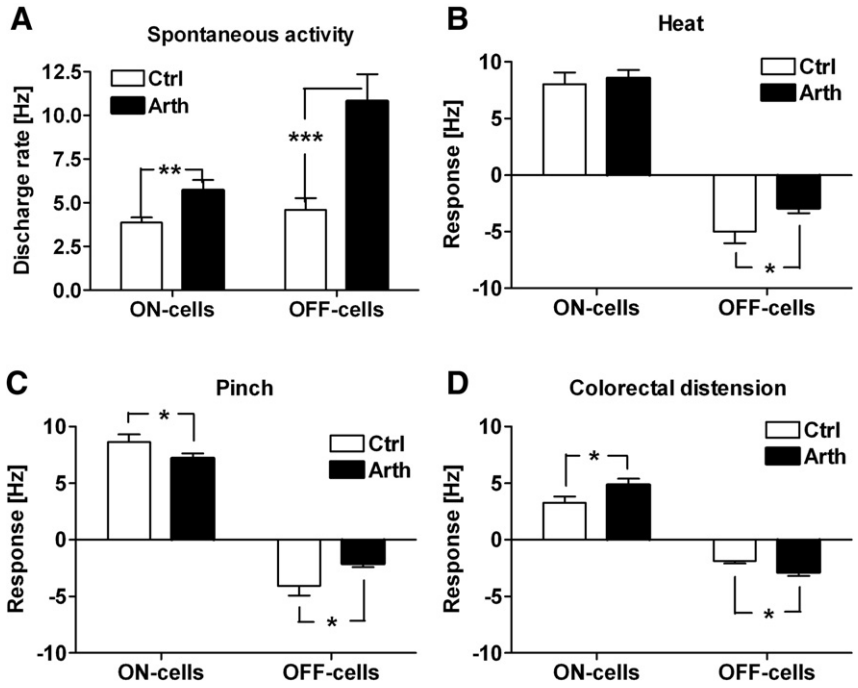

Fig. 2 – Response properties of ON- and OFF-cells of the RVM in control (Ctrl) and arthritic (Arth) animals. A) Spontaneous discharge rate. B) Response to noxious heating of the hind paw skin (ipsilateral to the inflamed knee joint in arthritic animals). C) Response to noxious pinch of the tail. D) Response to noxious visceral stimulation (colorectal distension). The error bars represent S.E.M. (n=14–23 in arthritic groups and n=22–34 in control groups). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ (t-test).

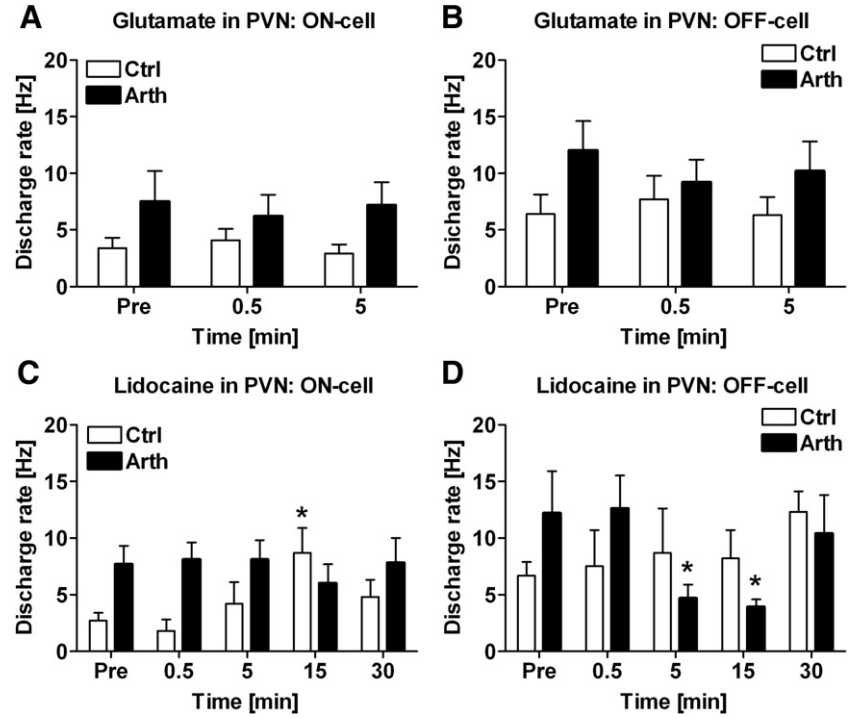

Fig. 3 – Mean spontaneous discharge rates of ON- and OFF-cells of the RVM in control (Ctrl) and arthritic (Arth) animals following microinjection of glutamate or lidocaine into the hypothalamic paraventricular nucleus (PVN). A) Effect of glutamate on discharge rate of ON-cells (n_{Ctrl}=18, n_{Arth}=11). B) Effect of glutamate on discharge rate of OFF-cells (n_{Ctrl}=9, n_{Arth}=6). C) Effect of lidocaine on discharge rate of ON-cells (n_{Ctrl}=8, n_{Arth}=8). D) Effect of lidocaine on discharge rate of OFF-cells (n_{Ctrl}=4, n_{Arth}=5). The error bars represent S.E.M. The Y-axis shows the time elapsed from the microinjection of glutamate or lidocaine. Pre = before injection. $^{*}P<0.05$ (Dunnett's test; Reference: the corresponding pre-injection rate).

2. Results

2.1. Behavioral characterization of arthritis

All animals in the arthritic group developed a clear swelling of the treated knee joint and all of them gave a vocalization response to a minor extension and flexion of the affected limb by the experimenter, whereas untreated control animals had no obvious swelling in the knee joint and they did not vocalize when the limb was moved.

2.2. Behavioral assessment of spinal antinociception induced by the PVN

Behaviorally, spinal nociception was assessed by determining the latency of the limb withdrawal response evoked by noxious heating of the hind paw. Saline, glutamate (50 nmol) or lidocaine (4%/0.5 μl) was microinjected into the PVN to study the phasic and tonic regulation of spinal nociception in arthritic animals versus controls. Administration of these compounds in the PVN had a significant effect on the heat-evoked hind-limb withdrawal latency ($F_{2,26}=45.3$, $P<0.0001$): when compared with saline, glutamate induced a significant prolongation (antinociception) and lidocaine a decrease (pronociception) of the withdrawal latency (Fig. 1 A). These modulatory effects by glutamate or lidocaine in the PVN were not significantly different between arthritic and control animals ($F_{1,26}=0.14$).

Naloxone (5.0 μg) was administered intrathecally to study the potential contribution of spinal opioid receptors to the antinociceptive action induced by glutamate in the PVN. Intrathecal administration of naloxone did not attenuate the antinociceptive effect induced by glutamate in the PVN of arthritic or control animals (Fig. 1 B). Intrathecal administration of naloxone alone had no effect in controls but it increased the withdrawal latency in arthritic animals.

Atipamezole, an α_2-adrenoceptor antagonist (5.0 μg), was administered intrathecally to study the involvement of spinal α_2-adrenoceptors in the antinociceptive action induced by administration of glutamate in the PVN. In control animals, atipamezole reversed the antinociceptive action of glutamate in the PVN, while atipamezole alone had no significant effect (Fig. 1 C). In arthritic animals, in contrast, atipamezole did not influence the glutamate-induced antinociception, whereas atipamezole alone induced a significant prolongation of the withdrawal latency (Fig. 1 C).

To study the role of spinal 5-HT_{1A} receptors in antinociception induced by glutamate in the PVN, WAY-100635 (3.0 μg), a 5-HT_{1A} receptor antagonist, was administered intrathecally. In control but not arthritic animals the antinociceptive action induced by glutamate in the PVN was reversed by intrathecal administration of WAY-100635. When administered alone, WAY-100635 had no significant influence on the limb withdrawal latency in arthritic or control animals (Fig. 1 D).

2.3. Response characteristics of ON- and OFF-cells of the RVM

The RVM provides a potential link for mediating the pain-regulatory effect from the PVN to the spinal dorsal horn. In this study, we focused on assessing response properties of the presumably pronociceptive ON-cells and antinociceptive OFF-cells in the RVM. The number of RVM cells tested quantitatively was 37 (23 ON- and 14 OFF-cells) in arthritic animals and 56 (34 ON- and 22 OFF-cells) in controls. The receptive fields of ON- and OFF-cells were typically wide covering all extremities and the whole body. The distribution in the number of ON- and OFF-cells was not significantly different between arthritic and control animals (Fisher's exact test).

2.4. Spontaneous discharge rate of RVM cells

The spontaneous discharge rate of ON- and OFF-cells in the RVM was significantly increased by arthritis ($F_{1,187}=32.6$, $P<0.0001$; Fig. 2 A). The spontaneous discharge rate of OFF-cells was significantly higher than that of ON-cells ($F_{1,187}=17.2$, $P<0.0001$), and this difference was significantly larger in arthritic animals ($F_{1,187}=9.5$, $P<0.003$).

2.5. Peripherally evoked responses of RVM cells

When assessing the peripherally evoked response of ON- and OFF-cells, the noxious stimuli were applied to the non-inflamed area distal to the arthritic knee joint (heat), the tail (pinch) or the viscera. The magnitude of the excitatory ON-cell response evoked by noxious heating of the hind paw skin was not significantly different between arthritic and control animals,

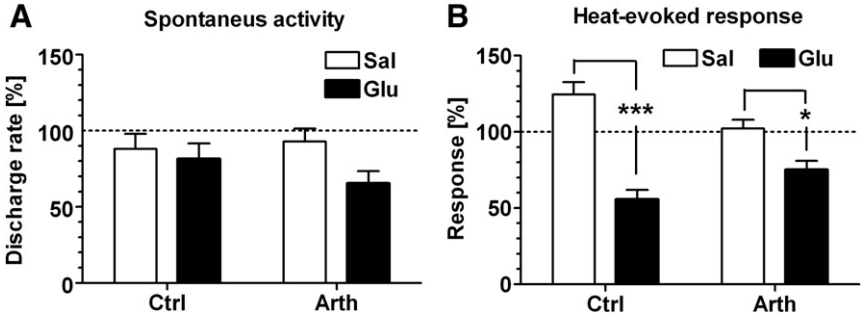

Fig. 4 – Mean changes in spontaneous discharge rates (A) and noxious heat-evoked responses (B) of spinal dorsal horn wide-dynamic range (WDR) neurons following injection of glutamate (Glu) or saline (Sal) into the hypothalamic paraventricular nucleus (PVN) in control (Ctrl) and arthritic (Arth) animals. 100% represents the corresponding pre-injection value. The error bars represent S.E.M. ($n_{Sal}=5$, $n_{Glu}=7$). $^{*}P<0.05$, $^{***}P<0.005$ (t-test).

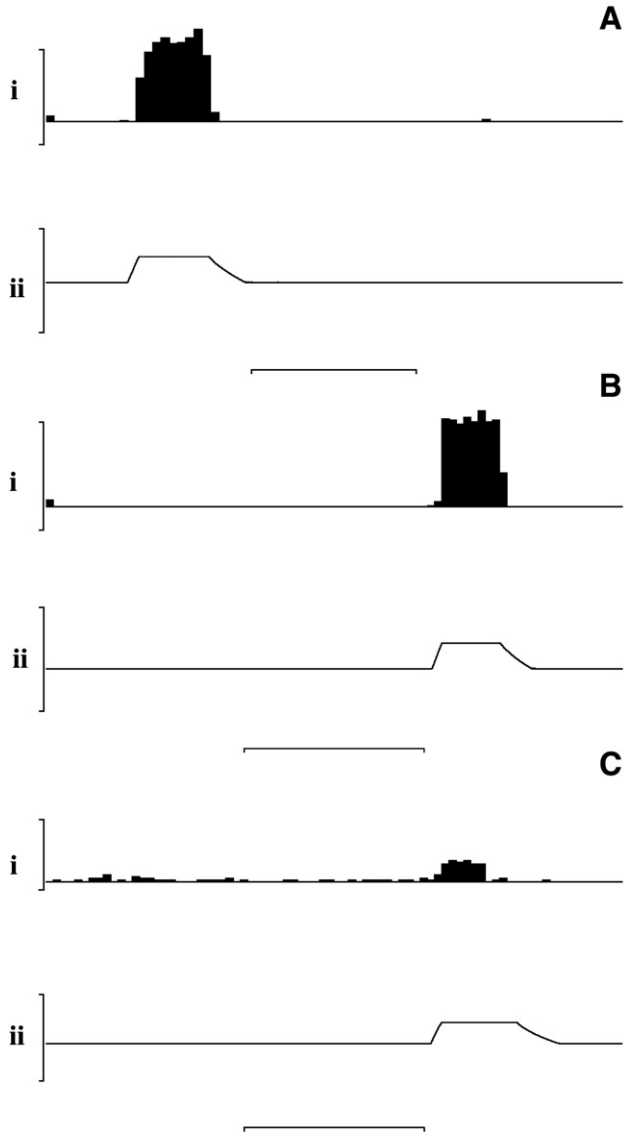

Fig. 5 – Responses of a spinal dorsal horn WDR neuron to noxious heat stimulation of the hind paw in a control animal before PVN injections (A), 30 s after injection of saline in the PVN (B), and 30 s after injection of glutamate in the PVN (C). i: neuronal response, ii: heat stimulus that starts from the baseline temperature of 35 °C and reaches the peak temperature of 54 °C. Vertical calibration bar for i represents 50 Hz and the horizontal one 25 s.

whereas the magnitude of the inhibitory OFF-cell response evoked by noxious heating was reduced in arthritis (Fig. 2 B). The magnitudes of the ON- and OFF-cell responses evoked by noxious tail pinch were reduced in arthritic animals (Fig. 2 C). In contrast, the magnitudes of colorectal distension-induced responses of ON- and OFF-cells were slightly but significantly increased in arthritis (Fig. 2 D).

2.6. *Effects of glutamate or lidocaine administration in the PVN on discharge rates of RVM cells*

The spontaneous discharge rate of ON- and OFF-cells of the RVM was assessed following microinjection of glutamate or lidocaine in the PVN to study arthritis-induced changes in descending modulation of nociception originating in the PVN and relaying through the RVM. Glutamate in the PVN had no significant influence on the discharge rate of ON-cells in arthritic animals ($F_{2,32}=0.5$) or controls ($F_{2,53}=0.6$; Fig. 3 A). Neither did glutamate in the PVN influence the spontaneous discharge rate of OFF-cells in arthritic ($F_{2,26}=0.5$) or control animals ($F_{2,17}=2.1$; Fig. 3 B).

Lidocaine in the RVM had no influence on the discharge rate of ON-cells in arthritic animals ($F_{4,39}=1$), whereas it increased ON-cell activity in controls ($F_{4,39}=3.9$, $P<0.02$; Fig. 3 C). Following lidocaine administration in the PVN, OFF-cell activity was decreased in arthritic animals ($F_{4,24}=5.0$, $P<0.01$), but not changed in controls ($F_{4,19}=1.2$; Fig. 3 C).

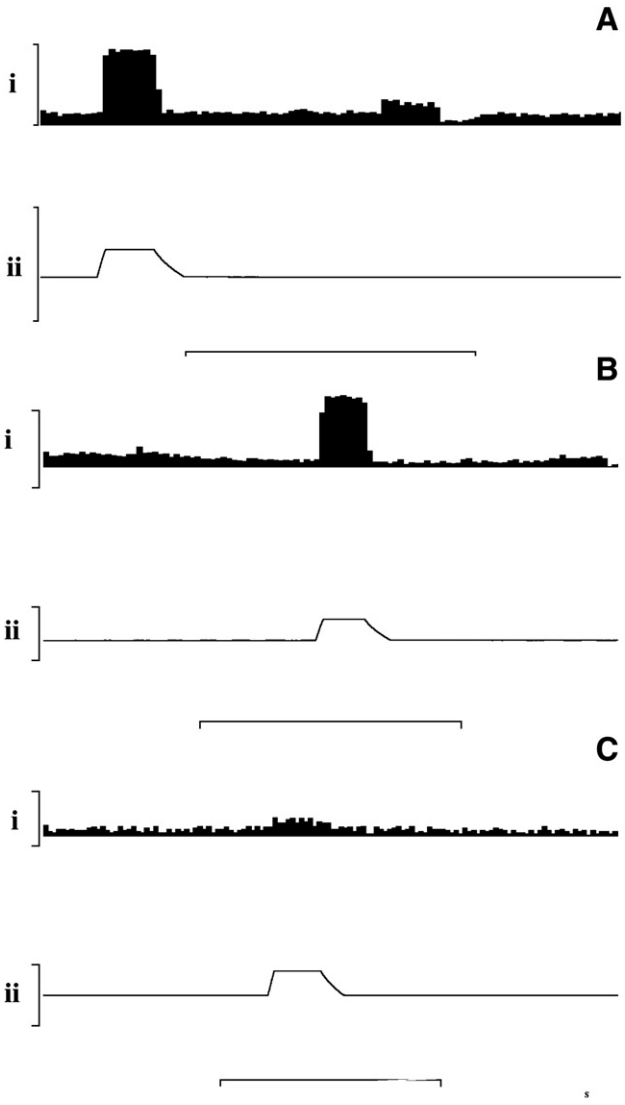

Fig. 6 – Responses of a spinal dorsal horn WDR neuron to noxious heat stimulation of the hind paw in an arthritic animal before PVN injections (A), 30 s after injection of saline in the PVN (B), and 30 s after injection of glutamate in the PVN (C). i: neuronal response, ii: heat stimulus that starts from the baseline temperature of 35 °C and reaches the peak temperature of 54 °C. Vertical calibration bar for the trace i represents 50 Hz, and the horizontal one 25 s.

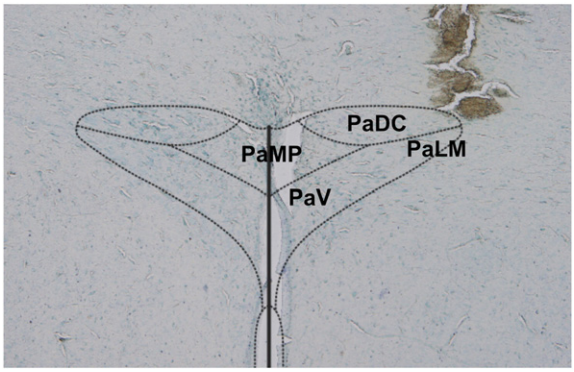

Fig. 7 – A photographic example of a microinjection site in the hypothalamus. Pa: paraventricular nucleus, PaV: ventral part of Pa, PaLM: lateral magnocellular part of Pa, PaDC: dorsal cap of Pa, PaMP: medial parvocellular part of Pa.

2.7. *Spinal dorsal horn WDR neurons*

Effect of glutamate in the PVN on spinal dorsal horn WDR neurons was determined to exclude the possibility that the PVN-induced modulation of spinal nociceptive reflex responses was rather due to suppression of spinal motor than sensory responses. While arthritis produced a significant increase in the baseline spontaneous discharge rate of WDR neurons ($P<0.05$, *t*-test), glutamate in the PVN failed to produce a significant suppression of the spontaneous discharge rate of WDR neurons ($F_{1,20}=2.02$; Fig. 4 A), independent of the experimental group ($F_{1,20}=0.73$). Heat-evoked responses of spinal dorsal horn WDR neurons were significantly decreased by glutamate in the PVN when compared with the effect of saline ($F_{1,20}=9.8$, $P<0.001$; Figs. 4 B, 5 and 6), and this glutamate-induced spinal antinociceptive effect was not significantly different between arthritic and control animals ($F_{1,20}=0.05$).

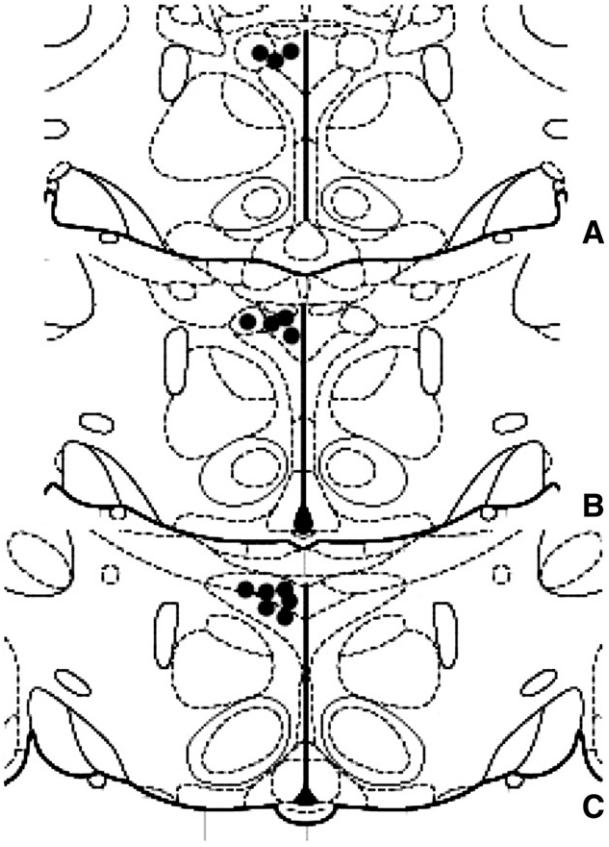

Fig. 8 – Microinjection sites in the PVN. The anteroposterior distance from the interaural line is 7.28 mm for section A, 7.20 mm for section B, and 7.09 mm for section C. Each symbol represents cannula locations in one to four animals.

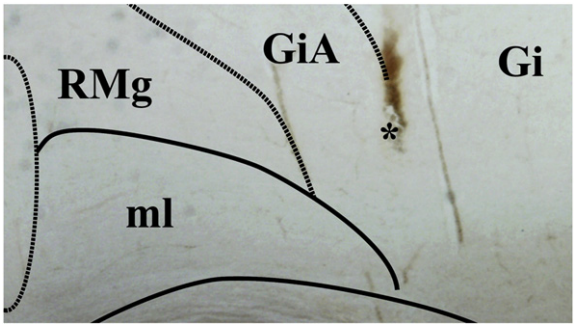

Fig. 9 – A photographic example of a recording site in the medulla (marked with an asterisk). Gi: gigantocellular nucleus, GiA: alpha part of Gi, RMg: raphe magnus nucleus, ml: medial lemniscus.

2.8. *Injection and recording sites*

Figs. 7 and 8 show microinjection sites in the PVN, and Figs. 9 and 10 show recording sites in the RVM. Based on the estimated spread of the currently used injection volume of 0.5 μl (Myers, 1966), the injections spread both to the magno- and parvocellular areas of the PVN and areas immediately adjacent to the PVN. The recording sites in the RVM were in the raphe magnus and the adjacent medial bulboreticular formation. In the spinal dorsal horn, recording sites were in the deep spinal dorsal horn as assessed from the depth of recording sites from the cord surface (400–1000 μm).

3. Discussion

3.1. *Influence of arthritis on the PVN-induced spinal antinociception and a potential relay in the RVM*

Glutamate in the hypothalamic paraventricular nucleus (PVN) suppressed and lidocaine in the PVN facilitated noxious heat-evoked spinal withdrawal responses in arthritic and control animals. This finding indicates that the PVN has a role in phasic and tonic suppression of spinal nociception in arthritic as well as control conditions. It is noteworthy that glutamate in the PVN suppressed not only a spinal withdrawal reflex but also the response of presumed pain-relay neurons in the spinal dorsal horn indicating that the PVN induced rather a true antinociceptive action than only a suppression of the motor expression of nociception. Moreover, the present results indicate that arthritis induces changes in firing rates of presumed pain-modulatory cells in the rostroventromedial medulla (RVM), a structure that

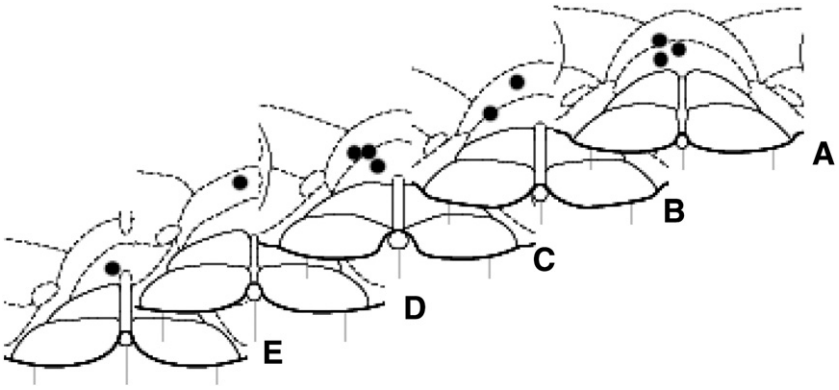

Fig. 10 – Recording sites marked with electrolytic lesions in the RVM. The anteroposterior distance from the interaural line is 1.92 mm for section A, 2.04 mm for section B, 2.16 mm for section C, 2.40 mm for section D and 2.64 mm for section E. Each symbol represents recording sites of one to five neurons.

receives efferent projections from the PVN (Holstege, 1987) and that is known to be an important relay for descending modulation of spinal nociception (Gebhart, 2004). Interestingly, arthritis-induced changes in spontaneous firing rates of pronociceptive ON- and antinociceptive OFF-cells of the RVM were likely to have opposite effects on spinal antinociception. This is indicated by the finding that the spontaneous activity of both pronociceptive ON-cells and antinociceptive OFF-cells was increased in arthritis. The concurrent promotion of descending pro- and antinociceptive influence from the RVM of arthritic animals may contribute to the observations that the baseline nociception of arthritic animals outside of the inflamed region, as indicated by the withdrawal response to heating of the hind paw distal to the inflamed joint, was not significantly different from that in controls. Previous results indicate that during the first hours, inflammation leads to enhanced descending facilitation from the RVM whereas during a later phase the net descending effect from the RVM is inhibition (Terayama et al., 2000). This finding suggests that the pronociceptive influence of arthritis might have been stronger in the present study if the experiments had been performed within the first few hours, instead of several days after induction of arthritis. It should also be noted that the currently used injection volume of 0.5 μl may have spread to areas adjacent to the target area in the PVN and therefore, the present results do not allow excluding the possibility that brain areas adjacent to the PVN contribute to the present findings.

Glutamate in the PVN failed to influence discharge rates of RVM cells in arthritic or control animals. This finding suggests that the RVM may not have a critical role in mediating phasic antinociception induced by PVN-stimulation. On the other hand, lidocaine in the PVN increased firing rates of pronociceptive ON-cells in control animals and decreased firing of antinociceptive OFF-cells in arthritic animals. This finding suggests that the PVN in a tonic and dissociative fashion drives the RVM and that the drive is changed by arthritis. The net descending effect of the PVN-induced tonic drive need not, however, be changed by arthritis, since the PVN-induced tonic suppression of pronociceptive RVM ON-cells in control animals may have an equal effect on spinal nociception as the PVN-induced tonic facilitation of antinociceptive RVM OFF-cells. In line with this proposal, the behavioral results indicated that lidocaine in the PVN had an equal spinal pronociceptive effect in arthritic and control animals. These findings are in line with the hypothesis that the RVM is involved in mediating tonic PVN-induced modulation of spinal nociception.

The magnitudes of pinch- and heat-evoked responses of RVM cells were decreased in arthritis. It should be noted, however, that in this study pinch and heat were applied to the skin area outside of the inflamed joint. Therefore, sustained nociceptive barrage from the inflamed joint may have attenuated concurrent nociceptive signals evoked by pinch and heat stimulation of the healthy skin area. In line with this proposal, this type of a phenomenon that is also called diffuse noxious inhibitory controls (Le Bars et al., 1979) is known to be effective in arthritis (Calvino et al., 1987). Although the RVM is not involved in mediating diffuse noxious inhibitory controls (Bouhassira et al., 1993), the RVM receives ascending nociceptive signals from the spinal dorsal horn, a structure that is influenced by diffuse noxious inhibitory controls (Le Bars et al., 1979). Responses to noxious visceral stimulation, in contrast, were slightly enhanced in arthritis. Possibly the converging cutaneous receptive fields of spinal neurons mediating visceral nociception from the colorectal area are large enough to receive and summate sustained nociceptive signals from the inflamed joint which might explain enhanced visceral responses.

Previous studies have shown that a number of pathophysiological models such as prolonged noxious thermal stimulation, opioid withdrawal, mustard oil-induced neurogenic inflammation and spared nerve injury model of neuropathy produce hypersensitivity that is associated with increased activity of pronociceptive ON-cells in the RVM (Bederson et al., 1990; Gonçalves et al., 2007; Kincaid et al., 2006; Morgan and Fields, 1994; Xu et al., 2007) and that may, in some conditions, be accompanied by a decreased activity of antinociceptive OFF-cells (Gonçalves et al., 2007). In the present study, arthritis increased activity of both pro- and antinociceptive RVM cells. Arthritis failed to produce a significant change in the limb withdrawal evoked by heating the paw distal to the inflamed joint; this was expected based on the arthritis-induced changes in discharge properties of RVM cells. Together, the results are in line with the hypothesis that ON- and OFF-cells of the RVM have a role in modulation of spinal nociception in various

pathophysiological as well as control conditions (Fields et al., 2006), although the magnitude of contribution and the pattern of firing rate changes may vary depending on the experimental condition.

3.2. *Spinal neurotransmitter receptors involved in the PVN-induced antinociception*

In control animals, antinociception induced by glutamate in the PVN was reversed by spinal administration of a 5-HT_{1A} receptor antagonist and an α_2-adrenoceptor antagonist, whereas the effect of an opioid receptor antagonist on the PVN-induced antinociception was not significant. This finding indicates that under physiological conditions serotoninergic raphe-spinal and descending noradrenergic pathways acting on spinal 5-HT_{1A} and α_2-adrenoceptors, respectively, are involved in mediating the PVN-induced spinal antinociceptive action. This is in line with previous results indicating that the PVN has efferent connections to various pain-modulatory nuclei in the brainstem, including the serotoninergic raphe magnus (Holstege, 1987; Swanson and Sawchenko, 1983) and that electrical or chemical stimulation of the RVM may inhibit nociception due to action on spinal 5-HT_{1A} receptors (el-Yassir and Fleetwood-Walker, 1990; Wei and Pertovaara, 2006). Efferent connections from the PVN directly to the noradrenergic locus coeruleus in the pons (Swanson and Sawchenko, 1983) provide a link for activation of descending noradrenergic pathways that contribute to the PVN-induced antinociception due to action on spinal α_2-adrenoceptors. Additionally, the PVN might recruit descending noradrenergic pathways through the RVM (Nuseir et al., 1999; Sim and Joseph, 1992). In line with earlier findings (Shiraishi et al., 1995; Yirmiya et al., 1990), the present results suggest that spinal opioid receptors do not have a critical role in the PVN-induced antinociception in control animals.

Unlike under control conditions, the contribution of spinal 5-HT_{1A} receptors to the PVN-induced antinociception was not significant in arthritic animals. Thus, arthritis induced a change in the contribution of the serotoninergic system to the PVN-induced antinociception. While spinal administration of an α_2-adrenoceptor or opioid receptor antagonist alone had no significant effect on pain-related behavior in control animals, these compounds produced a significant modulatory action in inflamed animals. Paradoxically, the changes produced by an α_2-adrenoceptor or opioid receptor antagonist alone were prolongations of the limb withdrawal latency. A plausible explanation for the paradoxically increased withdrawal latency by the receptor antagonists alone is removal of arthritis-induced noradrenergic and opioidergic feedback inhibition (Pertovaara, 2006; Yaksh, 2006) and a consequent increase in the sustained nociceptive barrage from the inflamed joint that led to a central suppression of heat-evoked responses from the cutaneous test site in the hind paw; i.e., spinally administered α_2-adrenoceptor and opioid receptor antagonists may have enhanced sustained joint pain and consequently, diffuse noxious inhibitory controls (Calvino et al., 1987) that suppressed concurrent nociception elsewhere. Due to significant actions by the α_2-adrenoceptor and opioid receptor antagonists alone, the present results do not allow concluding whether the contribution of spinal noradrenergic or opioid receptors to the PVN-induced antinociceptive effect is changed in arthritis.

3.3. *Spinal neurotransmitters mediating descending antinociception from the PVN versus other hypothalamic areas*

Interestingly, while the present results indicate that spinal 5-HT_{1A} receptors and α_2-adrenoceptors are involved in mediating the descending antinociceptive effect from the PVN in control conditions, earlier results indicate that these monoaminergic receptors mediate descending antinociception also from the lateral hypothalamus (Holden and Naleway, 2001; Holden et al., 2005). In contrast, while some earlier (Shiraishi et al., 1995; Yirmiya et al., 1990) and the present results indicate that spinal opioid receptors have only a minor, if any, role in the PVN-induced antinociception, the spinal antinociceptive effect induced by stimulation of the hypothalamic arcuate nucleus was reversed by spinal administration of an opioid receptor antagonist (Wang et al., 1990b).

3.4. *Conclusions*

The PVN has a phasic and tonic descending antinociceptive influence in arthritic as well as control animals. The RVM may contribute to the relay of descending influence from the PVN. Arthritis induced a dual change in the baseline activity and the PVN-induced tonic drive of pro- and antinociceptive cells of the RVM. Due to these dual arthritis-induced changes that produced opposite actions, the net effect of RVM cells in the control of baseline nociception or in the relay of tonic inhibitory influence from the PVN may remain the same, although the roles of pro- and antinociceptive cells vary between the arthritic and control conditions. Recent studies indicate that vasopressin (Yang et al., 2006) or oxytocin (e.g., Condés-Lara et al., 2006; Miranda-Cardenas et al., 2006) released from hypothalamo-spinal neurons have an important role in the PVN-induced antinociception. These findings indicate that direct action by descending axons of hypothalamic neurons in the spinal dorsal horn may alone be sufficient to induce antinociception. The present results extend these findings by showing that descending serotoninergic and noradrenergic pathways acting on spinal 5-HT_{1A} receptors and α_2-adrenoceptors, respectively, may also contribute to the PVN-induced inhibition of spinal nociception in control conditions.

4. Experimental procedures

4.1. *Animals, anesthesia and ethical issues*

The experiments were performed in adult male Wistar Han rats with 250–300 g (Harlan Netherlands, Horst, Netherlands). The experimental protocol was approved by the Institutional Ethical Commission and followed the European Community Council Directive 86/609/EEC for the use of experimental animals. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

For the experimental surgery and electrophysiological sessions, anesthesia was induced by administering pentobarbitone (50 mg/kg, i.p.) and the anesthesia was maintained by infusing pentobarbitone (15–20 mg/kg/h, i.p.) when necessary. The level of anesthesia was frequently monitored by observing the size of

the pupils, the general muscle tone and behavioral responses to noxious pinching. Importantly, the anesthesia level was maintained in an identical fashion when studying control and arthritic animals. Therefore, a potential influence of anesthesia level, if any, was identical in control and arthritic groups. A warming blanket was used to maintain the body temperature within physiological range. At the completion of the experiment, animals received a lethal dose of pentobarbitone.

4.2. *Procedures for intrathecal and intracerebral microinjections*

For the insertion of the intrathecal cannula, a thin polyethylene cannula (PE-10, Becton Dickinson & Co., Sparks, MD) was inserted into the lumbar subarachnoid space as described in detail elsewhere (Størkson et al., 1996). The intrathecally inserted catheter was then fixed through a layer of superficial muscles, tunneled rostrally and made to appear through the skin in the occipital region. Upon recovery from anesthesia, 10 μl of 2% lidocaine hydrochloride, followed by 10–15 μl of saline was given through the catheter – with the help of a 50 μl-Hamilton microsyringe (Hamilton Inc., Reno, NV) – to verify if it was indeed spinally located. Only rats that developed reversible symmetrical paralysis of both hind limbs and tail after the injection of lidocaine were used in the experiments. Intrathecal cannula was inserted at least one week before actual experiments. Test-drugs were injected intrathecally at a volume of 5 μl using a 50 μl-Hamilton microsyringe, flushed afterwards with 10–15 μl of saline.

For intracerebral drug administration, the rats were placed in a stereotaxic frame and a stainless steel guide cannula (26 gauge; Plastics One, Roanoke, VA) was implanted in the brain according to the coordinates of the atlas by Paxinos and Watson (1998). The tip of the guide cannula was positioned 1 mm above the desired injection site in the PVN (AP, 7.2 mm; LM, 0.2 mm; DV, 7.9 mm to the interaural line). After the guide cannula was fixed into the skull using a dental screw and dental cement, a dummy cannula was inserted into the guide cannula and the top was closed. Animals were allowed to recover from surgery for one week before testing.

Test-drugs were administered in the PVN through a 33-gauge injection cannula (Plastics One) inserted into and protruding 1 mm beyond the tip of the guide cannula. The microinjection was made using a 1.0-μl-Hamilton syringe connected to the injection cannula by a polyethylene catheter (PE-10). The injection volume was 0.5 μl and therefore, the spread of the injected drugs within the brain was at least 1 mm (Myers, 1966). The efficacy of injection was monitored by watching the movement of a small air bubble through the tubing. The injection lasted 30 s and the injection cannula was left in place for an additional 30 s to minimize the return of drug solution back to the injection cannula. Brain injection sites were histologically verified from post-mortem sections and plotted on standardized sections derived from the stereotaxic atlas of Paxinos and Watson (1998).

4.3. *Induction of arthritis*

The induction of arthritis was performed 7–14 days before the actual experiments as described in detail elsewhere (Ansah and Pertovaara, 2007). Briefly, 3% kaolin and 3% carrageenan (Sigma, St. Louis, MO, USA) were dissolved in distilled water and injected into the synovial cavity of the left knee joint at a volume of 0.1 ml. This model produces mechanical hyperalgesia with the onset of a few hours and a duration of up to 8 weeks (Radhakrishnan et al., 2003). In each animal, development of arthritis was verified 1–2 h prior to each experiment. Only those rats that vocalized every time after five flexion–extension movements of the knee joint were considered to have arthritis, and they were included in the arthritis group. Untreated control animals did not vocalize to any of the five consecutive flexion–extension movements of the knee joint.

4.4. *Behavioral assessment of nociception*

The rats were habituated to the experimental conditions by allowing them to spend 1–2 h daily in the laboratory during two to three days preceding any testing. For assessing nociception in unanesthetized animals, radiant heat-induced latency of paw withdrawal was determined using radiant heat equipment (Plantar Test Device Model 7370, Ugo Basile, Comerio, Italy) as described in detail earlier (Hargreaves et al., 1988). Radiant heat was applied to the plantar skin of the hind limb ipsilateral to the inflamed knee joint and the PVN injection. In each drug treatment session, the withdrawal latency was assessed prior to drug treatment and at various interval following the intracerebral and intrathecal injections. At each time point, the measurement was repeated twice at an interval of 1 min and the mean of these values was used in further calculations. Cut-off time was 20 s. Since spinal transection does not abolish the heat-induced limb withdrawal (e.g., Kauppila et al., 1998), it is a spinally organized nociceptive reflex, although it is modulated by brainstem–spinal pathways in intact animals. Therefore, the heat-induced limb withdrawal provides a method for determining spinal nociception and its supraspinal modulation in behaving animals and also under anesthesia (e.g., Luukko et al., 1994).

4.5. *Recording of neuronal responses in the rostroventromedial medulla (RVM)*

RVM neurons provide a potential relay for descending influence from the RVM. Therefore, we studied the response properties of RVM neurons and the modulation of their activity by the PVN in control and arthritic animals. For electrophysiological recordings of neurons in the RVM, anesthesia was induced and continued as described above, and the animal was placed in a standard stereotaxic frame according to the atlas of Paxinos and Watson (1998). The skull was exposed and a hole was drilled for placement of a recording electrode in the RVM. The desired recording site in the RVM was 1.8–2.3 mm posterior from the ear bar, 0.0–0.5 mm lateral from the midline, and 8.9–10.7 mm ventral from the dura mater. Single neuron activity was recorded extracellularly with lacquer-coated tungsten electrodes (tip impedance 3–10 MΩ at 1 kHz) and then amplified and filtered using standard techniques. Data sampling was performed with a computer connected to a CED Micro 1401 interface and using Spike 2 software (Cambridge Electronic Design, Cambridge, U.K.).

Actual recordings of RVM neurons did not start until the animal was under light anesthesia; i.e., the animals gave a

brief withdrawal response to noxious pinch, but the pinch did not produce any longer lasting motor activity, nor did the animals have spontaneous limb movements. RVM neurons were classified based on their response to noxious heating (54 °C) of the hind paw with a feedback-controlled Peltier device (LTS-3 Stimulator, Thermal Devices Inc., Golden Valley, MN; Wilcox and Giesler, 1984), as described below. For detection of a heat-evoked limb withdrawal concurrently with the neuronal response, a piezoceramic movement detector (Siemens Elema Ab., Medicinsk Teknik, Solna, Sweden) of low weight (<0.5 g) was taped on the skin overlying the hamstring muscle in the mid thigh level of the stimulated hind limb and the movement of the limb measured with it as described earlier (Hämäläinen et al., 1996). For classification of RVM neurons, the scheme developed earlier (reviewed by Fields et al., 2006) was adapted. Briefly, neurons giving an excitatory heat-evoked response that was associated with a hind-limb withdrawal were considered to be pronociceptive ON-cells, those giving an inhibitory response that was associated with a limb withdrawal were considered to be antinociceptive OFF-cells (Fig. 11). Neurons showing no or only a negligible (<10%) change in their discharge rates as a response to noxious stimulation were considered to be NEUTRAL-cells which were not studied further in this investigation. If a neuron could not be classified it was not included in the study. Classification of RVM neurons was not attempted unless the noxious test stimulus induced a hind-limb withdrawal reflex.

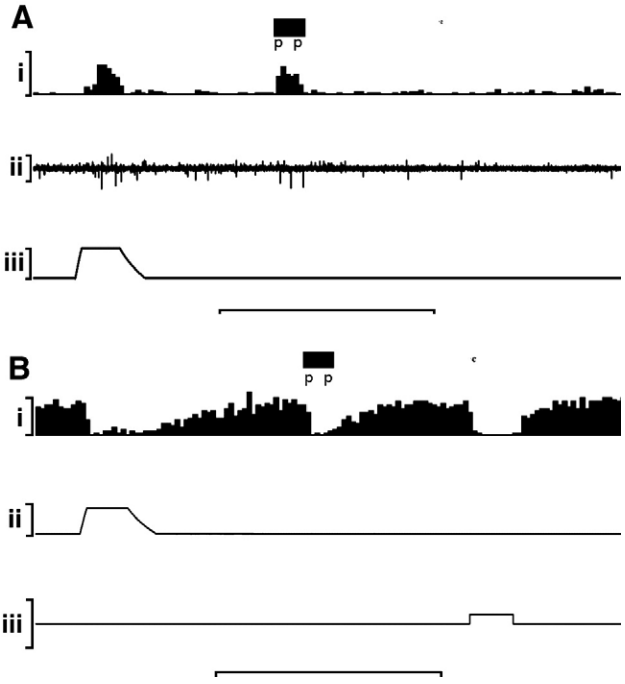

Fig. 11 – Examples of original recordings of RVM cells. A) ON-cell in a control animal. B) OFF-cell in an arthritic animal. In A, i shows the neuronal response, ii the withdrawal response in the hind limb, and iii the noxious heat stimulus applied to the hind paw. In B, i shows the neuronal response, ii the noxious heat stimulus, and iii the noxious visceral stimulus (colorectal distension). In both graphs, P–P indicate the duration of the noxious tail pinch. The vertical calibration bar for neuronal response represents 10 Hz in A and 20 Hz in B. In both graphs, the baseline temperature of the heat stimulus is 35 °C and the peak stimulus temperature 54 °C. In B, colorectal distension is applied at an intensity of 80 mmHg. The horizontal calibration bar represents 50 s in A and 40 s in B.

Characterization of the response properties of an RVM cell consisted of the following assessment performed successively: 1. Spontaneous activity. 2. Response to heating of the hind paw ipsilateral to the treated knee with a Peltier device (LTS-3 Stimulator; a heat ramp rising at the rate of 10 °C/s from the baseline temperature of 35 °C to the peak temperature of 54 °C and peak duration of 10 s). 3. Response to pinching of the tail for 5 s by a surgical clamp that produced painful sensation when applied to the hand of the experimenter. 4. Response to colorectal distension (CRD) at a noxious intensity (80 mmHg; Ness et al., 1991) and duration of 10 s. CRD was produced by inflating with air a 7–8 cm flexible latex balloon inserted transanally into the descending colon and rectum. The pressure in the balloon was controlled by an electronic device (Anderson et al., 1987).

When analyzing responses of RVM neurons to peripheral stimulation, the baseline discharge frequency recorded during a corresponding period just before the stimulation was subtracted from the discharge frequencies determined during stimulation; i.e., positive values represent excitatory responses evoked by peripheral stimulation and negative ones inhibitory responses.

The animals used in recordings had a guide cannula for drug administrations into the PVN. Electrophysiological experiments were performed one to two weeks after fixation of the guide cannula to the skull, as described above. After determining the responses of an RVM neuron to peripheral stimulation, the phasic modulation of the discharge rate of RVM neurons by the PVN was assessed by microinjecting glutamate (50 nmol in 0.5 μl) into the PVN using methods described above. The discharge rate of the RVM cells was followed up to 5 min after the injection of glutamate. Thereafter, tonic control of the RVM by the PVN was assessed by microinjecting lidocaine (4% in 0.5 μl) into the PVN and following the discharge rate of RVM neurons up to 30 min.

4.6. *Recording of neuronal responses in the spinal dorsal horn*

To exclude the possibility that the PVN-induced modulation of spinal reflex responses is rather due to action on spinal motor than sensory neurons, we determined the PVN-induced effect on responses of wide-dynamic range (WDR) neurons of the spinal dorsal horn. One to two weeks before the recordings of spinal dorsal horn neurons, a chronic guide cannula was inserted to the PVN as described above. Following induction of anesthesia with pentobarbitone (50 mg/kg i.p. followed by 15–25 mg/kg/h or more, if required according to continuous observation of the anesthesia level), a laminectomy was performed at the level of T12–L2 vertebrae. The dura was removed and the spinal cord was covered with warm mineral oil. Two spinal clamps, one rostral and one distal to the laminectomy, were used to stabilize the preparation. Data sampling methods

were the same as with the RVM recordings (see above). In the spinal dorsal horn, search and classification of spinal units was performed as described in detail elsewhere (Pertovaara et al., 2001). Only wide-dynamic range (WDR) neurons activated by innocuous stimulation (brush) and giving a differential response to heat stimulation within nociceptive range (46–54 °C) were studied further. All the WDR neurons included in the study had their receptive fields in the plantar skin of the hind paw. The recording depth from the spinal cord surface was 0.4–1.0 mm.

When assessing the PVN-induced modulation of the response of a spinal dorsal horn neuron, the noxious test stimulus was a heat ramp applied from a Peltier device (LTS-3 Stimulator). The stimulus started from the baseline temperature of 35 °C and ascended to the peak temperature of 54 °C at a rate of 10 °C/s. The duration of the peak temperature was 10 s. The response to heat was determined 5 min prior to and 30 s after the injection of saline or glutamate (50 nmol in 0.5 μl) into the PVN. The magnitude of the response before the injection was considered the reference response (100%) for each neuron. The order of testing glutamate or saline was varied between the neurons and the interval between testing the effects of glutamate and saline on the same neuron was at least 5 min. The interval between testing different neurons in the same animal was at least 30 min.

4.7. *Drugs*

The opioid receptor antagonist naloxone hydrochloride and the 5-HT_{1A} receptor antagonist WAY-100635 were purchased from Sigma (St. Louis, MO, USA), while the α_2-adrenoreceptor antagonist atipamezole was obtained from Orion Pharma Inc. (Turku, Finland). The intrathecal doses of naloxone, WAY-100635 and atipamezole were chosen based on our previous investigations showing that at the dose range used these receptor antagonists alone had no significant effects on nociception in control or neuropathic animals (Pertovaara and Wei, 2003, in press; Wei and Pertovaara, 2006). It should be noted that unlike many other α_2-adrenoreceptor antagonists, atipamezole does not bind to 5-HT_{1A} receptors (Pertovaara et al., 2005). Sodium pentobarbitone, glutamate and physiological saline were obtained from Orion Pharma Inc. (Espoo, Finland), and the local anesthetic, lidocaine, was obtained from Astra (Södertälje, Sweden).

4.8. *Course of the behavioral study*

One to two weeks following induction of the arthritis and at least one week following insertion of the intrathecal catheter and the guide cannula for PVN injections, the efficacy of PVN-induced phasic and tonic modulation of spinal nociception was determined by assessing the effect of glutamate and lidocaine in the PVN on the heat-evoked spinal withdrawal reflex in unanesthetized arthritic and control animals. Physiological saline was used for control injections and untreated animals were used as control animals. In these experiments, the latency of the withdrawal response was assessed 30 s, 5 min, 15 min and 30 min following the injection. The latency measured 30 s after glutamate injection and 15 min after lidocaine injection was used in further calculations, since the maximum effects of the studied compounds are obtained at these time points. The interval between behavioral assessments of glutamate-, lidocaine- or saline-induced effects was at least two days and the order of testing different compounds was varied between the animals.

Assessment of spinal neurotransmitter receptors mediating the descending antinociceptive influence induced by glutamate in the PVN was also assessed one to two weeks following induction of arthritis. In these experiments, one of the three receptor antagonists studied (atipamezole, WAY-100635 or naloxone) was administered intrathecally immediately following the assessment of the pre-drug latency. The effect of the receptor antagonist alone on the withdrawal latency was assessed 10 min following its intrathecal administration. At this time point, all the studied receptor antagonists should have their maximum effects. Glutamate (50 nmol) was microinjected into the PVN about 13 min following the intrathecal injection of the receptor antagonist. To assess possible reversal of the glutamate-induced antinociception by the spinally administered receptor antagonist, the heat-evoked withdrawal latency was again determined 30 s after injection of glutamate into the PVN; i.e., the potential reversal of PVN-induced antinociception was determined about 14–15 min following the intrathecal injection of the receptor antagonist. When testing different receptor antagonists in the same animal, the interval between testing sessions was at least two days. The order of testing different receptor antagonists was varied between the animals. Each animal participated in 1–3 behavioral testing sessions. At the end of the experiment, the animals were given a lethal dose of pentobarbitone and the brains were removed for histological verification of the injections sites.

4.9. *Course of the electrophysiological study*

Electrophysiological recordings of RVM neurons or spinal dorsal horn neurons were performed under pentobarbitone anesthesia in different animals one to two weeks following the induction of arthritis and at least one week following the insertion of the guide cannula for PVN injections. In RVM recordings, the response properties of the neurons were assessed by determining spontaneous activity and response to noxious heating of the skin, tail pinch and CRD. Then, the change in spontaneous activity of RVM neurons following successive microinjections of glutamate and lidocaine at a 15 min interval into the PVN was assessed as described in detail above. Search for the next neuron to be studied started about 30 min after the testing of the previous one was completed. At the end of the recording session, electrolytic lesions were made in the recording sites, the animals were given a lethal dose of pentobarbitone and the brains were removed for histological verification of the recording and injection sites.

In recordings of spinal dorsal horn WDR neurons, the heat-evoked response was determined before and 30 s after injection of glutamate or saline into the PVN; i.e., testing was performed at the time point when glutamate has its maximum effect. The interval between testing the saline and glutamate in the same neurons was at least 5 min, and the interval between testing different neurons in the same animal was at least 15 min. At the end of the recording session, the animals were given a lethal dose of pentobarbitone and the brain removed for histological verification of the injection site.

4.10. *Statistics*

Two-way analysis of variance (ANOVA) followed by Dunnett's test (comparisons between three or more groups) or *t*-test (comparisons between two groups) were used in statistical assessment of the data. The differences in the incidence of various types of RVM neurons were analyzed using Fisher's exact test. $P<0.05$ was considered to represent a significant difference.

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Chapter 2.3.2

Pinto-Ribeiro F, Ansah OB, Almeida A, Pertovaara A.

The caudal ventrolateral medulla (CVLM) as a relay centre of the antinociceptive effect of the paraventricular nucleus of the hypothalamus (PVN) in an animal model of monoarthritis

(Manuscript submitted)

The caudal ventrolateral medulla (CVLM) as a relay centre of the antinociceptive effect of the paraventricular nucleus of the hypothalamus (PVN) in an animal model of monoarthritis

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Keywords: Caudal ventrolateral medulla; Arthritic pain; Descending modulation; Hypothalamus; Spinal nociception

Abbreviations:

PVN - paraventricular nucleus of the hypothalamus;

CVLM - caudal ventrolateral medulla

PAG – periaqueductal gray matter

WDR - wide-dynamic range neurones

CONT – control animals/controls

ARTH – arthritic animals/arthritis

CRD – colorectal distension

GLU – glutamate

LIDO – lidocaine

NPS – noxious peripheral stimulation

HPA – hypothalamus-pituitary-adrenal glands axis

Abstract

In this work the role of the caudal ventrolateral medulla (CVLM) upon pain modulation was studied in SHAM and arthritic (ARTH) rats using electrophysiological recordings of neuronal activity in the CVLM. As the CVLM participates in the regulation of homeostasis, the effect of the activation of the paraventricular nucleus of the hypothalamus (PVN) upon its activity was also assessed. The electrophysiological evaluation of CVLM neurones revealed the presence of three different types of cells, (+)/(-)/(=) cells, whose response pattern to acute noxious peripheral stimulation is similar to the ON-/OFF-/neutral cells of the rostral ventromedial medulla (RVM). Arthritis induced an increase in the spontaneous activity of CVLM (+) and (=) cells while it decreased (-) cell activity. Significant changes were also observed in (i) the proportion of (+)/(-)/(=) cells [increase in the total number of (-) cells responsive to cold paralleled by a decrease in (=) cells], and in (ii) the noxious-evoked response of both (+)/(-) CVLM neurones to different types of stimulation. Additionally, the PVN exerts an inhibitory downstream effect upon the pronociceptive CVLM (+) cells during noxious stimulation, but only in ARTH animals.

These results point to the occurrence of plastic changes in the CVLM and in the PVN-mediated descending circuitry due to chronic (arthritic) pain.

1. Introduction

The concept of how pain is perceived and modulated has slowly evolved over the last decades. Initially it was thought that the exacerbation of pain was a direct consequence of increased signalling of the ascending pathways and analgesia resulted from the activation of the descending pathways. At this time, the descending modulatory pain system was mainly associated with the periaqueductal gray matter (PAG)-rostral ventromedial medulla (RVM) system (Basbaum and Fields, 1984). The electrophysiological examination of RVM neurones revealed the existence of three cell classes (Fields and Heinricher, 1985) whose firing pattern "ON-"and "OFF-cells" upon peripheral noxious stimulation allowed their association with the facilitation and inhibition of nociception, respectively (Fields *et al.*, 1983; Heinricher *et al.*, 2009). These data were essential for demonstrating that the descending modulatory pain system alone was a dynamic system capable of either inhibiting or facilitating pain sensation (Ren and Dubner, 2002; Vanegas and Schaible, 2004). This concept was further reinforced by more recent studies showing that other brainstem areas are also involved in the descending positive and negative facilitation of nociceptive transmission, such as the dorsal reticular nucleus (DRt) and caudal ventrolateral medulla (CVLM), respectively (Lima and Almeida, 2002; Tavares and Lima, 2002; Heinricher *et al.*, 2009). While the DRt is activated mainly by noxious stimulation (Villanueva *et al.*, 1988) and facilitates acute, inflammatory (Almeida *et al.*, 1996; 1999) and neuropathic (Sotgiu *et al.*, 2008) pain, the CVLM produces profound analgesia after electrical stimulation and glutamate administration (Gebhart and Ossipov 1986; Janss and Gebhart 1986), and seems to tonically inhibit spinal nociceptive neurones (Tavares *et al.*, 1997).

It has been proposed that the CVLM modulation of nociceptive transmission is mediated through direct and indirect pathways to the spinal cord, mechanisms that appear to be specific to the nature of the noxious stimuli and the spinal cell where it originates from (Tavares and Lima, 2002). Also important is its involvement in indirectly mediating the hypoalgesia due to increased blood pressure, through a CVML-A5-spinal cord circuitry (Tavares *et al.*, 1997). In addition, the CVLM and the nucleus tractus solitarius are part of a three-way loop that involves supraspinal reciprocal projections with the paraventricular nucleus of the hypothalamus (PVN) (Krukoff *et al.*, 1994; Kawano and Masuko, 1996). The PVN is a major integration centre of several circuits, including the stress response through the hypothalamus-pituitary-adrenals axis (Armario, 2006; Lightman and Young, 1988) and pain modulation (Pinto-Ribeiro *et al.*, 2008; Delatorre *et al.*, 2009). In a previous study, we assessed the modulatory effect of the PVN upon RVM cells in

normal in arthritic animals (Pinto-Ribeiro *et al.*, 2008) and demonstrated that although the RVM may not have a crucial role in mediating phasic antinociception induced by PVN stimulation, this nucleus tonically modulates the activity of RVM nociceptive neurones in both control and arthritic conditions. However, it is not known if other components of the supraspinal pain system are recruited by the PVN in phasic pain control during normal or chronic pain conditions.

Our purpose in this study was to: (i) characterize the electrophysiological profile of CVLM neurones, both at rest and after acute noxious stimulation in normal rats; (ii) analyse eventual changes in CVLM neuronal activity after the induction of monoarthritis; (iii) investigate a potential role of the PVN as a modulator of CVLM neuronal activity, as an alternative pathway for PVN-mediated stress-induced analgesia.

2. Experimental procedures

2.1. Animals, anaesthesia and ethical issues

The experiments were performed in adult male Wistar Han rats with 175–250g (Harlan Netherlands, Horst, Netherlands). The experimental protocol was approved by the Institutional Ethical Commission and followed the European Community Council Directive 86/609/EEC for the use of experimental animals. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

For the experimental surgery and electrophysiological sessions, anaesthesia was induced by administering pentobarbitone (50 mg/kg, i.p.) and maintained by infusing pentobarbitone (15–20 mg/kg/h, i.p.) when necessary. The level of anaesthesia was frequently monitored by observing the size of the pupils, the general muscle tone and behavioural responses to noxious pinching. Importantly, the anaesthesia level was maintained in an identical fashion when studying control and arthritic animals to avoid a potential influence of this factor amongst groups. A warming blanket was used to maintain the body temperature within physiological range. At the completion of the experiment, animals received a lethal dose of pentobarbitone.

2.2. Recording of neuronal responses in the caudal ventrolateral medulla (CVLM)

In order to perform electrophysiological recordings of the activity of CVLM neurones after being anaesthetized (as described above), the animals were placed in a standard stereotaxic frame. The skull was exposed and a hole was drilled for placement of a recording electrode in the CVLM

(4.08 mm posterior to interaural, 2.0–2.2 mm lateral from the midline, and 10.0 mm ventral from the dura mater) according to the atlas of Paxinos and Watson (2005) **(Fig. 1)**. Single neurone activity was recorded extracellularly with lacquer-coated tungsten electrodes (tip impedance 3–10 MΩ at 1 kHz) and then amplified and filtered using standard techniques. Data sampling was performed with a computer connected to a CED Micro 1401 interface and using Spike 2 software (Cambridge Electronic Design, Cambridge, U.K.).

Recording of CVLM neurones was started after the animal was under light anaesthesia; i.e., the animals gave a brief withdrawal response to noxious pinch, but the pinch did not produce any longer lasting motor activity, nor did the animals have spontaneous limb movements. CVLM neurones were classified based on their response to noxious heating (54 °C) of the hind paw with a feedback-controlled Peltier device (LTS-3 Stimulator, Thermal Devices Inc., Golden Valley, MN; Wilcox and Giesler, 1984), as described below. For detection of a heat-evoked limb withdrawal concurrently with the neuronal response, a piezoceramic movement detector (Siemens Elema Ab., Medicinsk Teknik, Solna, Sweden) of low weight (< 0.5 g) was taped on the skin overlying the hamstring muscle in the mid thigh level of the stimulated hind limb and the movement of the limb measured with it as described earlier (Hämäläinen *et al.*, 1996). For the functional classification of CVLM neurones, the scheme developed earlier for the RVM (Fields *et al.*, 1983) was adapted.

Briefly, neurones displaying an increase in firing rate evoked by heat stimulation of the tail, associated with a hind-limb withdrawal, were considered to be positive/excitatory (+) neurones to that stimulus, while those decreasing its activity were considered to be negative/inhibitory (-) neurones **(Fig. 2)**. Neurons displaying no or only a negligible (< 10%) change in their discharge rates as a response to noxious stimulation were considered to be neutral (=) cells, which were not studied further in this investigation. If a neuron could not be classified it was excluded from the study. Classification of CVLM neurones was not attempted unless the noxious test stimulus induced a hind-limb withdrawal reflex.

Characterization of the response properties of CVLM cells consisted of the following assessments performed successively: (i) Spontaneous activity; (ii) Response to heating of the hind paw ipsilateral to the arthritic or SHAM knee with a Peltier device (LTS-3 Stimulator; a heat ramp rising at the rate of 10 °C/s from the baseline temperature of 35 °C to the peak temperature of 54 °C and peak duration of 10 s); (iii) Response to pinching of the tail for 5 s by a surgical clamp

that produced painful sensation when applied to the hand of the experimenter; (iv) Response to colorectal distension (CRD) at a noxious intensity [80 mmHg (Ness *et al.*, 1991) and duration of 10 s]. CRD was produced by inflating with air a 7–8 cm flexible latex balloon inserted transanally into the descending colon and rectum; the pressure in the balloon was controlled by an electronic device (Anderson *et al.*, 1987); (v) Response to cold stimulation of the hind paw ipsilateral to the treated knee with a Peltier device (LTS-3 Stimulator; a heat ramp decreasing at the rate of 10 °C/s from the baseline temperature of 35 °C to the peak temperature of 4 °C and peak duration of 10 s).

When analyzing responses of CVLM neurons to peripheral stimulation, the baseline discharge frequency recorded during a corresponding period just before the stimulation was subtracted from the discharge frequencies determined during stimulation; i.e., positive values represent excitatory responses evoked by peripheral stimulation and negative ones inhibitory responses.

The animals used in recordings had a guide cannula for drug administration into the PVN (see section 2.3). Electrophysiological experiments were performed one to two weeks after fixation of the guide cannula to the skull, as described above. After determining the responses of a CVLM neurone to peripheral stimulation, the phasic modulation of the discharge rate of CVLM neurones by exciting PVN neurones was assessed by microinjecting glutamate (50 nmol in 0.5 μL) into the PVN using methods described above. The discharge rate of the CVLM cells was followed up to 5 min after the injection of glutamate. All results from the drug administration were plotted against the values obtained for the same time points after saline (SAL) injection in the PVN.

2.3. Procedures for intracerebral microinjections

For intracerebral drug administration, the rats were placed in a stereotaxic frame and a stainless steel guide cannula (26 gauge; Plastics One, Roanoke, VA) was implanted in the brain according to the coordinates of the atlas by Paxinos and Watson (2005). The tip of the guide cannula was positioned 1 mm above the desired injection site in the PVN (AP, 7.2 mm; LM, 0.2 mm; DV, 7.9 mm to the interaural line) or the CVLM. After the guide cannula was fixed into the skull using a dental screw and dental cement, a dummy cannula was inserted into the guide cannula and the top was closed. Animals were allowed to recover from surgery for one week before testing.

Test-drugs were administered in the PVN through a 33-gauge injection cannula (Plastics One, Bilaney, Germany) inserted into and protruding 1 mm beyond the tip of the guide cannula. The

microinjection was made using a 1.0-μL-Hamilton syringe connected to the injection cannula by a polyethylene catheter (PE-10; Plastics One, Bilaney, Germany). The injection volume was 0.5μL and therefore, the spread of the injected drugs within the brain was expected to be 1 mm (Myers, 1966). The efficacy of injection was monitored by watching the movement of a small air bubble through the tubing. The injection lasted 20s and the injection cannula was left in place for an additional 30s to minimize the return of drug solution back to the injection cannula. Brain injection sites were histologically verified from post-mortem sections and plotted on standardized sections derived from the stereotaxic atlas of Paxinos and Watson (2005).

2.4. Induction of arthritis

The induction of arthritis (ARTH) was performed 7–14 days before the actual experiments, as described in detail elsewhere (Ansah and Pertovaara, 2007). Briefly, 3% kaolin and 3% carrageenan (Sigma, St. Louis, MO, USA) were dissolved in distilled water and injected into the synovial cavity of the left knee joint at a volume of 0.1 mL. This model produces mechanical hyperalgesia, which begins just in a few hours after surgery and extends up to 8 weeks (Radhakrishnan *et al.*, 2003). In each animal, development of arthritis was verified 1–2 h prior to each experiment. Only those rats that vocalized every time after five flexion–extension movements of the knee joint were considered to have arthritis, and they were included in the arthritis group. An experimental group used as SHAM animals were also injected with 0.1 mL saline in the synovial cavity of the left knee joint. SHAM animals did not vocalize to any of the five consecutive flexion–extension movements of the knee joint.

2.5. Drugs

Sodium pentobarbitone, glutamate and physiological saline were obtained from Orion Pharma Inc. (Espoo, Finland).

2.6. Course of the electrophysiological study

Electrophysiological recordings of CVLM neurones were performed under pentobarbitone anesthesia in different animals one to two weeks following the induction of arthritis and at least one week following the insertion of the guide cannula for PVN injections. In CVLM recordings, the response properties of the neurons were assessed by determining spontaneous activity and response to noxious heating of the skin, tail pinch and CRD. Then, the change in spontaneous

activity of CVLM neurones following successive microinjections of glutamate at 30 min interval into the PVN was assessed as described in detail above. Search for the next neuron to be studied started about 30 min after the testing of the previous one was completed. At the end of the recording session, electrolytic lesions were made in the recording sites, the animals were given a lethal dose of pentobarbitone and the brains were removed for histological verification of the recording and injection sites.

2.7. Statistics

The differences in the number of the different types of CVLM neurones between experimental groups (SHAM and ARTH) were analyzed using Fisher's exact test (Fig. 3). The differences in spontaneous activity between controls and arthritic animals were tested using a student *t*-test comparison (Fig. 4). The statistical assessment of differences in neuronal activity between experimental groups during the pharmacological treatments was assessed using two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test (Figs. 5,6). A value of $p<0.05$ was considered to represent a significant difference.

3. Results

3.1 Activity of CVLM neurones in control and arthritic animals

Our first aim was to investigate the response of CVLM cells to a noxious stimulus in basal conditions and a total number of 78 neurones were studied (**Table 1**). The application of a noxious heat stimulus to the hindpaw allowed us to identify three types of nociceptive responsive CVLM cells (**Fig. 3A**). A group of cells was excited after noxious peripheral stimulation (NPS) and was classified has excitatory (+) cells; the firing rate of a second group of neurones was decreased by NPS, which were thus classified as inhibitory (-) cells; finally, we observed no change in neuronal activity after NPS in a third group of neurones [(=)-cells]. The application of other modalities of cutaneous and visceral noxious stimuli resulted also in a similar change of activity pattern from CVLM cells, which presented increase [(+)-cells], decrease [(-)-cells] and no changes [(=)-cells] in their firing rates (**Figs. 3B,C,D; Table 1**). The spontaneous activity of these neurones was also consistent with the cell type, with (=) cells displaying the lowest firing rate followed by (+) cells and by (-) cells (**Fig. 4**). In an attempt to further correlate the activity of these cells to nociceptive processing, a second group of animals was subjected to arthritis and the activity of CVLM was again evaluated after several modalities of NPS. For this part of the study

the number of neurones studied was 77 (**Table 1**). After ARTH induction, the number of (+), (-) and (=) CVLM cells responding to noxious stimulation by heat (Chi-test, p=0.38), pinch (Chi-test, p=0.60) and colorectal distension (Chi-test, p=0.52) was identical to SHAM (**Figs. 3A-C**). However, an alteration in CVLM cell type ratio was observed between SHAM and ARTH animals after stimulation by cold (Chi-test, p=0.04) (**Fig. 3D**); although no difference was observed for (+) cells, the number of (-) neurones responsive to cold increased significantly, which was paralleled by a decrease in the (=) type (**Fig. 3D**). Additionally, arthritis exerted an overall strong effect upon cell activity ($ANOVA_{2w}$, $F_{2,18}$=3,9, p<0,022), as the spontaneous activity of CVLM neurones in ARTH animals was considerably altered (**Fig. 4**). We observed a significant increase in the spontaneous activity of (+) cells ($t_{(2.732)}$=0.007) and (=) cells ($t_{(2.173)}$=0.0329), which was paralleled by a decrease in (-) cells ($t_{(2.9)}$=0.006) firing rate (**Fig. 4**).

3.2 Noxious-evoked activity of CVLM neurones in control and arthritic animals

Interestingly, in ARTH animals, it is not only the spontaneous activity of CVLM neurones but also the noxious evoked activity of each cell type that is altered (**Fig. 5**). After the induction of arthritis, the heat-evoked activity was altered independently of the cell type; while the evoked activity of (+) cells decreased (n_{SHAM}=9 and n_{ARTH}=9), the opposite effect was observed in (-) cells (n_{SHAM}=24 and n_{ARTH}=24) [$ANOVA_{2w}$, ARTH, $F_{int(1,62)}$=60.39, p<0,0001; Bonferroni, (+) cells – CTRL X ARTH, p<0.05 and (-) cells – CTRL X ARTH, p<0.001] (**Fig. 5A**). After noxious mechanical ((+) cells, n_{SHAM}=18 and n_{ARTH}=18 and (-) cells, n_{SHAM}=19 and n_{ARTH}=19) and cold ((+) cells, n_{SHAM}=21 and n_{ARTH}=18 and (-) cells, n_{SHAM}=19 and n_{ARTH}=17) stimulation we observed a significantly increased activity of (+) neurones [$ANOVA_{2w}$, ARTH – mechanical, $F_{int(1,70)}$=36.55, p<0,0001; Bonferroni, (+) cells – CTRL X ARTH, p<0.001 and ARTH – cold, $F_{int(1,71)}$=33.93, p<0,0001; Bonferroni, (+) cells – CTRL X ARTH, p<0.001], respectively] but no changes were detected for (-) cells (**Figs. 5B,D**). Finally, visceral noxious-evoked activity ((+) cells, n_{SHAM}=22 and n_{ARTH}=22 and (-) cells, n_{SHAM}=21 and n_{ARTH}=18) is decreased in both CVLM cell types [$ANOVA_{2w}$, ARTH, $F_{(1,79)}$=33.91, p<0,0001; Bonferroni, (+) cells – CTRL X ARTH, p<0.01 and (-) cells – CTRL X ARTH, p<0.001] (**Fig. 5C**).

3.3 Activation of the PVN always decreases the activity of CVLM (+)-cells

In the evaluation of the effect of the PVN upon CVLM cells activity a total of 47 cells were studied. Pharmacological activation of the PVN with glutamate did not alter the spontaneous activity of

CVLM cells in both SHAM [(+) cells - $ANOVA_{rm}$, p=0.21 and (-) cells - $ANOVA_{rm}$, p=0.12] and ARTH [(+) cells - $ANOVA_{rm}$, p=0.76 and (-) cells - $ANOVA_{rm}$, p=0.51], although changes in evoked-responses were observed.

During noxious heat stimulation (**Fig. 6A**), the microinjection of glutamate in the PVN altered neuronal activity depending on the CVLM cell type ($F_{(1,36)}$=25.53, p=0.001) but this effect was not influenced by the presence of ARTH ($F_{(1,36)}$=1.724, p=0.2). Neuronal activity was significantly decreased in CVLM (+) cells in the ARTH group when compared to basal activity [$ANOVA_{2w}$, p=0.001; Bonferroni, SHAM, p>0.05 and ARTH, p<0.05], and when compared with SHAM [$ANOVA_{2w}$, p=0.001; Bonferroni, SHAM, p<0.01 and ARTH, p>0.05]. Additionally, (-) cell activity was increased only in the SHAM group [$ANOVA_{2w}$, p=0.001; Bonferroni, SHAM, p<0.05 and ARTH, p>0.05] (**Fig. 6A**).

Interaction between the induction of arthritis and cell-type was detected during noxious mechanical-evoked activity in the response of CVLM neurones [$ANOVA_{2w}$, mechanical-evoked response, $F_{int(1,28)}$=7.161, p=0.012] (**Fig. 6B**). Glutamate activation of the PVN significantly decreased the activity of CVLM (+) cells in the ARTH group when compared to basal evoked responses [(+) cells, $ANOVA_{2w}$, p=0.03; Bonferroni, (+) cells, p=0.02] and when compared with SHAM animals [SHAM, $ANOVA_{2w}$, p=0.04; Bonferroni, (+) cells, p=0.03]. Additionally, (-) cell activity was increased only in ARTH animals [$ANOVA_{2w}$, p=0.03; Bonferroni, (-) cells, p=0.004] (**Fig. 6B**).

Interaction between the induction of arthritis and cell-type was also detected after visceral stimulation [$ANOVA_{2w}$, CRD-evoked response, $F_{int(1,37)}$=5.38, p=0.026]. Glutamate activation of the PVN exclusively decreased (+) cell activity in ARTH when compared to basal evoked responses [ARTH, $ANOVA_{2w}$, p=0.0002; Bonferroni, (+) cells, p<0.05] and to SHAM animals [GLU, $ANOVA_{2w}$, p=0.026; Bonferroni, (+) cells, p<0.01] (Figure **6C**).

The influence of arthritis was not independent of CVLM cell type [$ANOVA_{2w}$, cold-evoked response, $F_{int(1,26)}$=33.25, p<0.0001] in cold-evoked responses after the administration of glutamate in the PVN. Again, activation of the PVN decreased (+) cell activity only in ARTH animals [ARTH, $ANOVA_{2w}$, p=0.03; Bonferroni, (+) cells, p=0.014] when compared to basal evoked responses, with no difference on (-) cell response for both groups (**Fig. 6D**). The evoked-responses of the CVLM cells was significantly altered, (+) cells decreased while (-) cells increased [GLU, $ANOVA_{2w}$,

p<0.0001; Bonferroni, (+) cells, p<0.001 and (-) cells, p<0.05] in ARTH animals when compared to SHAM (**Fig. 6D**).

4. Discussion

In this work we demonstrate for the first time the presence in the CVLM of nociceptive responsive neurones that display an activity pattern similar to the pronociceptive ON- [(+) cells] and antinociceptive OFF- [(-) cells] neurones that were previously observed in other descending pain modulatory areas, such as the RVM (Heinricher *et al.*, 2009) and the PAG (Heinricher *et al.*, 1987). Additionally, changes in neuronal activity after induction of arthritis indicate that the CVLM has different roles in the processing of different types of noxious stimulation. Finally, we propose that the PVN, as part of the HPA axis, acts as a compensatory mechanism to the sensitization process in arthritic animals at the supraspinal level since its activation systematically decreases the activity of CVLM ON-cells and mostly increases the activity of CVLM OFF-cells, but only when chronic pain is installed.

4.1. Technical considerations

This study presents two major constrains, one related to the ability to verify the exact coordinates of each cell recorded and a second associated with the fact that the electrophysiological analysis of activity between SHAM and ARTH were performed in two different sets of animals. The first constrain we consider to be unavoidable; the confirmation of the specific coordinates of each neurone recorded would imply the use of a single animal per recording which would then ethically compromise the study in terms of the number of animals needed. Since more than one cell was recorded by session, the exact coordinates (dorsoventral, lateromedial and rostrocaudal) of each recording site were carefully registered at the time of its recording and were later plotted in relation to the coordinates of the last cell recorded confirmed by the analysis of *post-mortem* slides of the brain.

As for the second constrain, animals from both the SHAM and the ARTH groups could have been implanted with multiple chronic electrodes in the CVLM at the beginning of the experiment. This procedure would have allowed the continuous recording of cell activity during not only the inflammatory process, but more importantly, during pain chronification. A continuous recording would provide detailed data about the time point at which neuronal activity patterns shift, as

shown by our study. Alternatively, in order to compensate, the number of cells we recorded in SHAM and ARTH was identical and the differences observed were significant.

4.2. Activity pattern of CVLM neurones

In the present work we show that CVLM neurones (i) change their firing pattern after noxious stimulation, (ii) undergo changes in their firing patterns in chronic pain (arthritis) confirming a clear involvement of this area in the modulation of nociception (Gebhart and Ossipov, 1986) and (iii) are modulated by the hypothalamus. In 1983, Fields and colleagues associated changes in the cell activity of the RVM to the modulation of nociceptive inputs. There is a strong similarity between what was observed for the RVM and our results in the CVLM. Neurones within the CVLM responded in three different ways after noxious heat stimulation: (i) a first group of cells, with medium/low basal activity, was excited during the TF test and started firing just before or at the beginning of the tail withdrawal reflex; (ii) a second group, with a steady firing pattern as basal activity, decreased its activity just prior or at the start of the tail flick reflex, and; (iii) a third group of neurones, with low basal activity, whose firing pattern was not changed after noxious heat stimulation (Heinricher *et al.*, 2009). Following the same rationale as for ON-, OFF and NEUTRAL-cells (Heinricher *et al.*, 2009), it is suggested that the CVLM (+), (-) and (=) cells have a presumptive descending pronociceptive, antinociceptive and no effect upon spinal nociceptive transmission. A direct involvement of the CVLM in spinal modulation is not surprising, as this area not only shares reciprocal projections with lamina I and II nociceptive neurones (Tavares and Lima, 2002) but also with the RVM (Colombari *et al.*, 2001) and the NTS (Kawano and Masuko, 1996), areas strongly involved in pain modulation at the spinal level (Mtui *et al.*, 1993; Fields *et al.*, 1995).

It was interesting to verify that although the analysis of noxious-evoked activity of CVLM cells was performed using a heat stimulus, these same cells responded to other modalities of noxious stimulation. According to Schnell and colleagues (2002), classifying cell type using one single type of noxious stimulation was insufficient to determine the response pattern to noxious stimulation. Although some RVM neurones behaved as neutral cells to noxious heat, their response to mechanical noxious stimulation was as a typical ON- or OFF-cell. In our work, we not only verified that heat-evoked neutral cells could respond to either or both mechanical, visceral and cold stimulation as (+)/(-)/(=), but also that this was true for the majority of the cells studied. In fact, in the CVLM only a few cells were responsive to only one type of noxious stimulation or

alternatively had a clean (+)/(-)/(=) profile for all stimuli types. The CVLM seems to be constituted by a heterogeneous population of nociceptive cell profiles suggesting that this area is capable of fine tuning when it comes to the discrimination of nociceptive modalities. Nonetheless, the type and number of cells responsive to each type of noxious stimulation does not seem to be differently distributed within the CVLM. The highest number of (+) responsive cells were associated with heat and mechanical noxious stimulation, whereas most cells were (=) to visceral stimulation and were either (=) or (-) following cold stimulation. Taking into account that a predominance of (+) cells over other types of cells indicates a facilitatory role, that (+) cells in the CVLM are mostly associated with heat and mechanical stimulation and that the CVLM participates in the stress response (Bienkowski and Rinaman, 2008), it is possible that these CVLM (+) cells nuclei are partly involved in the fight or flight response triggered by external factors. In parallel, a comparatively higher number of (-) cells for COLD stimulation also supports the development of a response towards survival since intense cold can be debilitating. On the other hand, the CVLM does not seem deeply involved in visceral pain, as (=) responsive cells predominate following this type of stimulus.

Finally, it is worth noting that the basal activity of (+) CVLM cells is considerably higher (two to three-folds) than what has been described for ON-cells in the RVM (Fields *et al.*, 1983). One possible explanation for this discrepancy is the level of anaesthesia, as deeper or lighter anaesthesia levels might have altered not only the spontaneous, but also the evoked activity of neurones involved in nociception. Another possibility is that since all cells were initially classified as (+), (-) or (=) according to their response to heat stimulation and considering that they may display different response patterns to the other types of noxious stimulation, it is possible that a (+) cell to heat stimulation would have a higher basal firing rate since it could be a (-) cell to either/or pinch, visceral or cold stimulation. Accordingly, the basal activity of both ON- and OFF-RVM atypical cells, although slightly lower than what was observed in the CVLM, was also higher (Schnell *et al.*, 2002) than originally reported (Fields *et al.*, 1983).

4.3. CVLM neuronal activity in monoarthritis

Substantial data support a role of the CVLM in descending antinociception (Tavares and Lima, 2002). Tonic descending inhibition decreased after lesions in the CVLM (Hall *et al.*, 1982; Gall et al., 1998) and the electrical or pharmacological stimulation of the CVLM produces a profound and long lasting analgesia (Gebhart and Ossipov, 1986). However, it has been stated by several

authors that the role of the CVLM under pathological conditions remains unstudied (Lumb, 2002; Tavares and Lima, 2002). The induction of monoarthritis resulted in pronounced changes in the spontaneous and noxious-evoked activity of CVLM cells as well as in the number of neurones that responded to a specific type of stimulation. It is also interesting to note that the arthritis-induced activity changes were different between cell types. The spontaneous activity of (+) cells is increased in ARTH animals while the opposite effect was observed for (-) cells, which suggests a possible pronociceptive role of the CVLM in pathological (arthritic) conditions. In fact, although previous studies have reported an antinociceptive role for the CVLM (Tavares and Lima, 2002), other studies pointed to a pronociceptive influence of the CVLM/lateral reticular nucleus region (Mansikka and Pertovaara, 1996; Marques-Lopes *et al.*, 2010).

The proportion of cells reactive to cold noxious stimulation is altered in arthritic rats with an increase in (-) cells, which is in accordance with a previous study where an increase in the number of OFF-cells in the RVM occurred after inflammation (Montagne-Clavel and Oliveras, 1994). More importantly, there seems to be a shift in the number of cold-responsive cells between (=) and (-) neurones. While analysing changes in the brainstem after inflammation, Miki and colleagues (2002) reported a similar effect for RVM cells; during continuous electrophysiological recordings of RVM neutral cells, these cells changed their response profile to ON- and OFF-cells after the induction of inflammation. Neutral cells have for long been discarded due to its lack of responsiveness during acute stimulation; however, there is a growing number of reports that view these cells as great candidates for the increased allodynic effect of former innocuous input (Neumann *et al.*, 1996) that involve mechanical and, specifically in this case, cold sensitivity.

Heat hyperalgesia (during sleep), spontaneous pain (resting time) and especially mechanical hyperalgesia (associated to movement) are common complaints in arthritic patients (Schaible *et al.*, 2009). Although evoked responses of CVLM neurones vary greatly between the type of stimulus applied, this area does not seem involved in heat hyperalgesia since heat stimulation induced a decrease in (+) and an increase in (-) activity, suggesting an antinociceptive role. Visceral stimulation evokes a similar response in (+) cells, but since (-) activity is also significantly decreased it is difficult to evaluate the final net effect of these changes. However, the CVLM might facilitate mechanical analgesia since (+) cell activity is significantly increased while (-) cells remain unchanged. Most remarkably, noxious-evoked activity after cold stimulation was similar to

mechanical, as (+) cells activity was increased and (-) cell activity remained unchanged. There is an apparent paradox between an increase in the number of (-) cold-responsive cells, while a marked increase in cold-evoked (+) cell activity occurred. It has been suggested that OFF-cells activation is prevalent in relation to ON-cell activation since OFF-cells are responsible not only for exciting more off-cells, but also for inhibiting on-cells (Fields *et al.*, 1991) but it is difficult to estimate this effect without recording several neurones at the same time.

4.4 Influence of the PVN upon CVLM neuronal activity

Overall, the activation of the PVN descending inhibitory control during noxious stimulation appears to target mainly CVLM (+) cells by consistently depressing their activity. These results are not unexpected since it has already been shown that in pathological conditions (neuropathic pain), although the activity of both ON- and OFF-cells is changed, the resulting hypersensitivity seems to be strongly associated with increased ON-cell activity (Gonçalves *et al.*, 2007). The PVN-mediated decrease in the CVLM (+) cells can be consider a pathway to counteract and compensate for the increased sensitivity induced by arthritis. As for CVLM (-) cells, their activity is only influenced (increased) by the PVN during noxious cold stimulation reinforcing the idea that cold stimuli seem to constitute an intense hyperalgesic input that recruits both types of modulatory neurones in the CVLM of arthritic animals.

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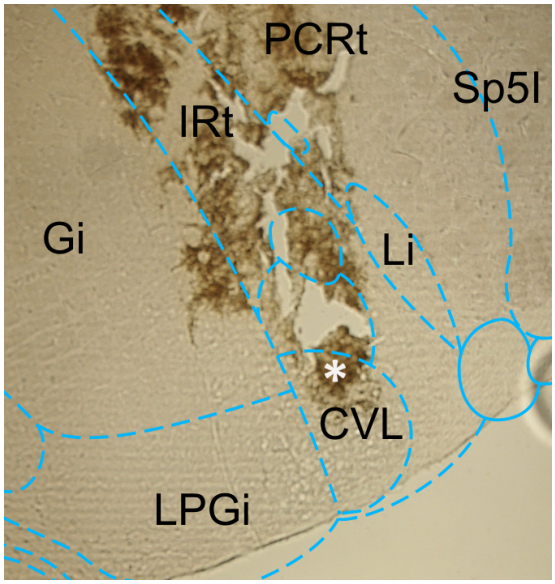

Figure 1 – A photographic example of a recording site in the caudal ventromedial medulla (CVL) (marked with an asterisk). (Gi – gigantocellular reticular nucleus, Li – linear nucleus of the medulla, IRt – intermediate reticular nucleus, Sp5l – lateral part of the trigeminal nucleus, PCRt – parvicellular reticular nucleus).

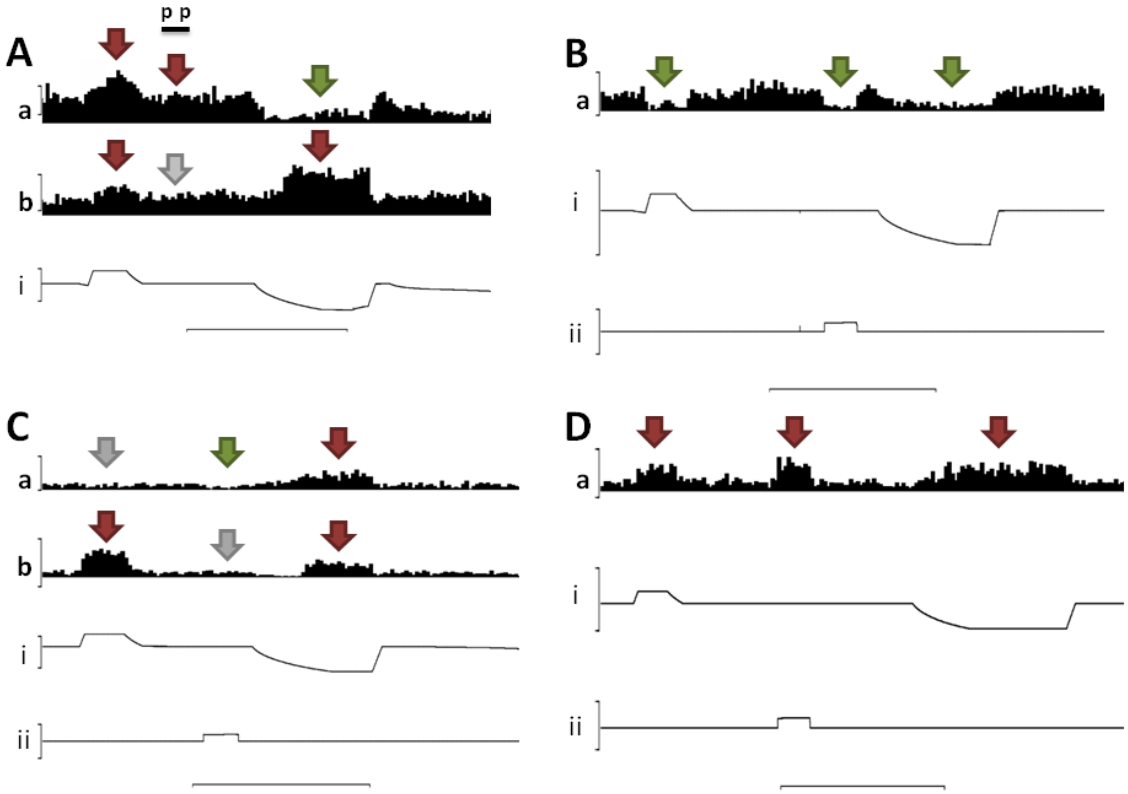

Figure 2. Examples of original recordings of CVLM cell responses to noxious peripheral stimulation. A) Simultaneous recording of two different CVLM neurones, neurone (a) increased its activity after noxious heat and mechanical stimulation, but decreased it after noxious cold stimulation of the hindpaw, whereas neurone (b) increased its activity after both noxious heat and cold stimulation of the hindpaw, but does not respond to noxious mechanical stimulation of the tail. B) Example of CVLM (-) cell, it decreases its activity after noxious colorectal distension and after heat and cold stimulation of the hindpaw. C) Simultaneous recording of two different CVLM neurones, neurone (a) is neutral to noxious heat stimulation however this cell decreases and increases its activity after noxious colorectal distension and cold stimulation of the hindpaw (respectively), whereas neurone (b) increased its activity after both noxious heat and cold stimulation of the hindpaw, but does not respond to colorectal distension. D) Example of a (+) cell, this neurones is responsive to all modalities of noxious stimulation applied, namely heat and cold stimulation of the hindpaw and colorectal distension. [*a,b* – represent different neurones within the same recording; i – represents the noxious heat and cold stimulus applied to the hind paw; ii – represents the noxious visceral stimulus (colorectal distension); *red arrows* – indicate an increase in neuronal activity; *green arrows* – indicate a decrease in neuronal activity; *gray arrows* – indicate no changes in neuronal activity; *P–P* indicates the duration of the noxious tail pinch (mechanical stimulation)]. The vertical calibration bar for neuronal response represents 10 Hz in A,B,D and 20 Hz in C. In all the graphs, the baseline temperature of the heat stimulus is 35 °C while the superior peak stimulus temperature is at 54°C and the inferior peak stimulus temperature at 4°C. In B, C and D colorectal distension was applied with an intensity of 80 mmHg. The horizontal calibration bar represents 50 s in D and 40 s in A, B and,C.

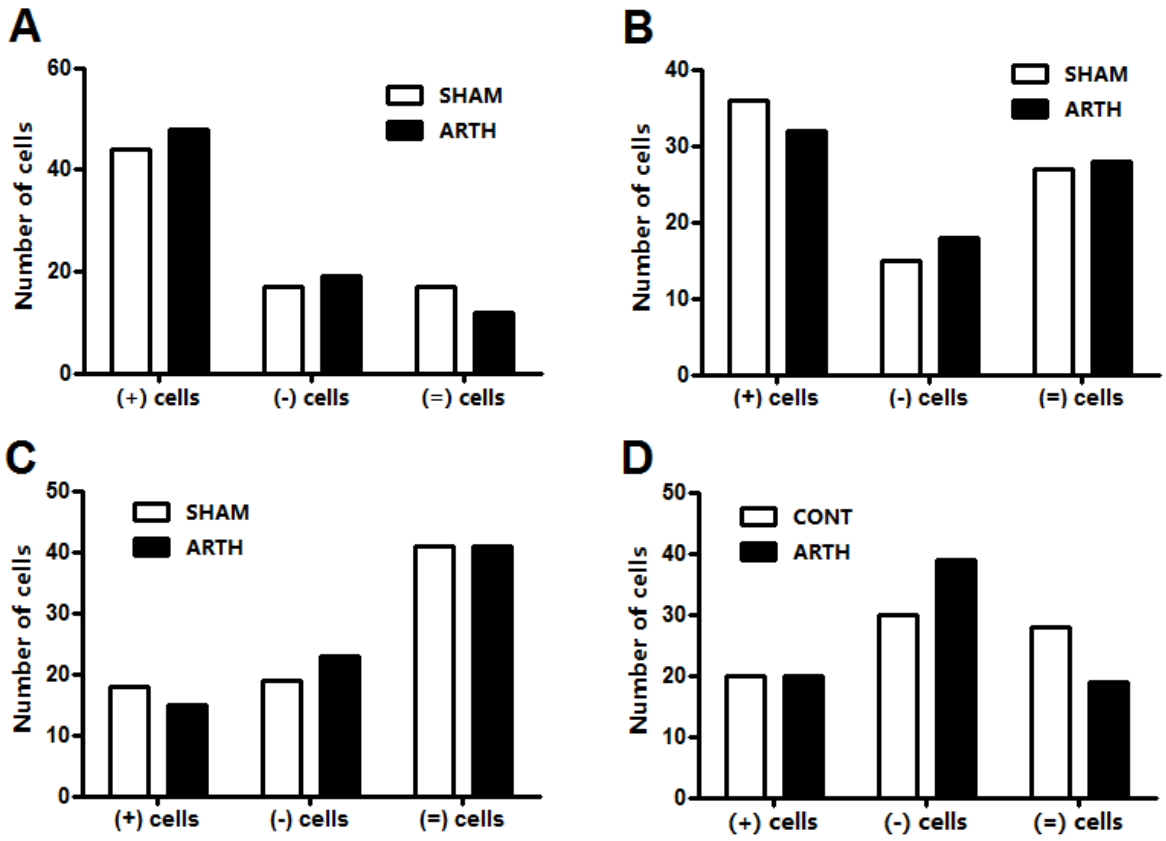

Figure 3 – Analysis of the firing pattern of CVLM cells in CONT and ARTH animals after noxious stimulation. Note that only noxious cold stimulation altered the (+)/(-)/(=) cell ratio of CVLM cell types, with a significant increase in the number of (-) cells and the opposite effect on (=) cells. (A – noxious heat stimuli to the hindpaw; B – noxious mechanical of the tail; C – noxious visceral stimulation; D – noxious cold stimuli to the hindpaw) (*p<0.05)

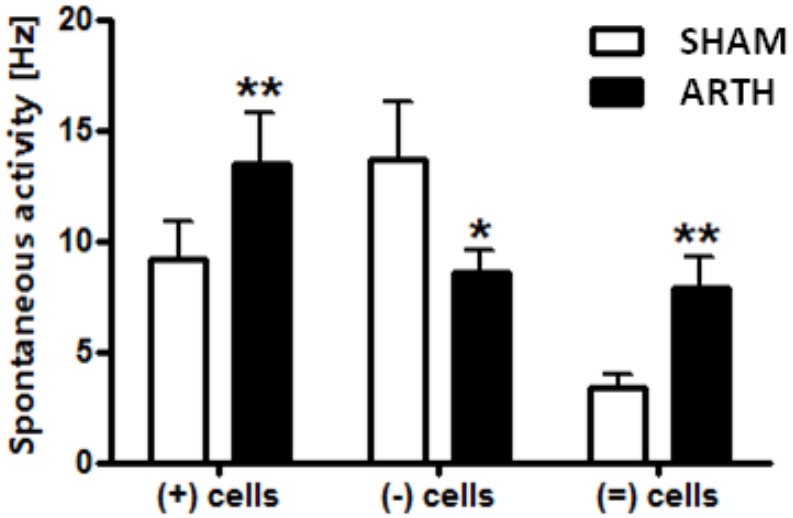

Figure 4 – Spontaneous activity of CVLM neurones in CONT and ARTH animals. Note that after the induction of arthritis the activity of CVLM neurones was significantly altered, with an increase in the firing rate of (+) and (=) cells being paralleled by a decrease in (-) cell activity. (*p<0.05, **p<0.01).

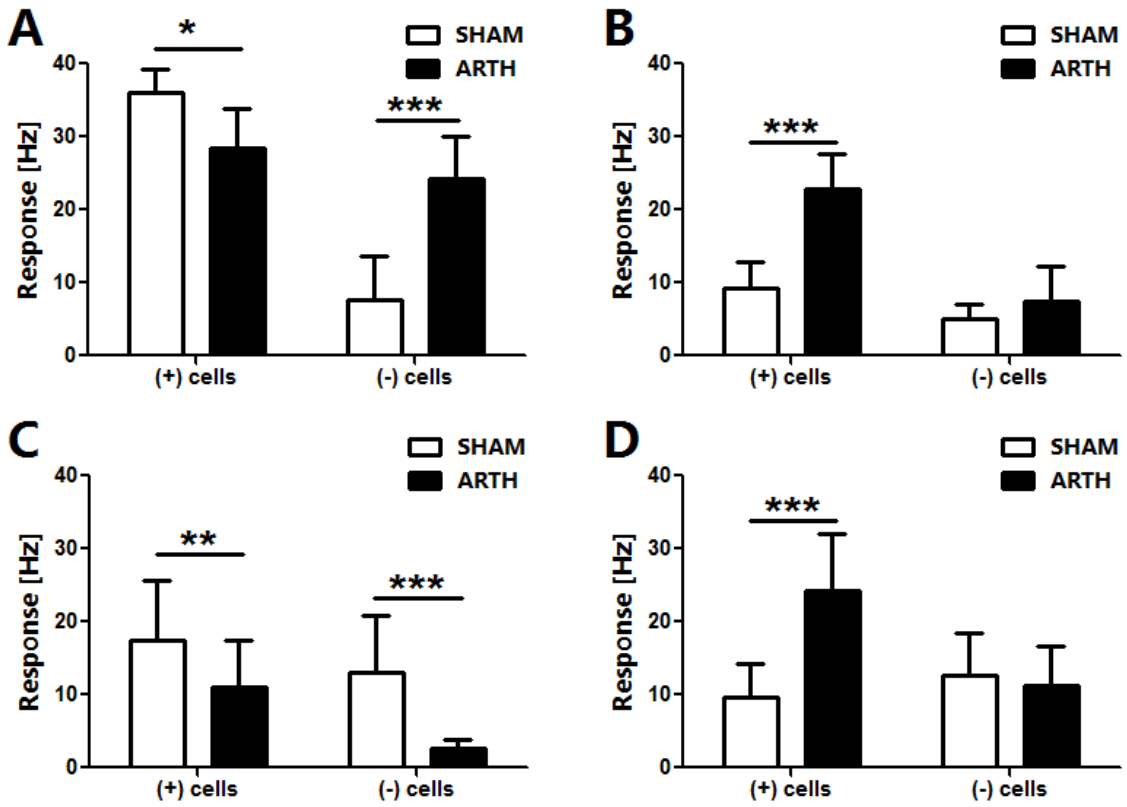

Figure 5 - Evoked response of CVLM (+) and (-) cells after the application of different modalities of noxious stimulation in SHAM and ARTH animals. Note the significant decrease of both (+) and (-) cells after heat stimulation, the increase of (+) cells after both mechanical and cold stimulation and the decrease of both (+) and (-) cells during visceral stimulation. (A – noxious heat stimulation to hind-paw; B – noxious mechanical stimulation of the tail; C – Noxious visceral stimulation; D – noxious cold stimulation of the hind-paw) (*p<0.05, **p<0.01, ***p<0.001)

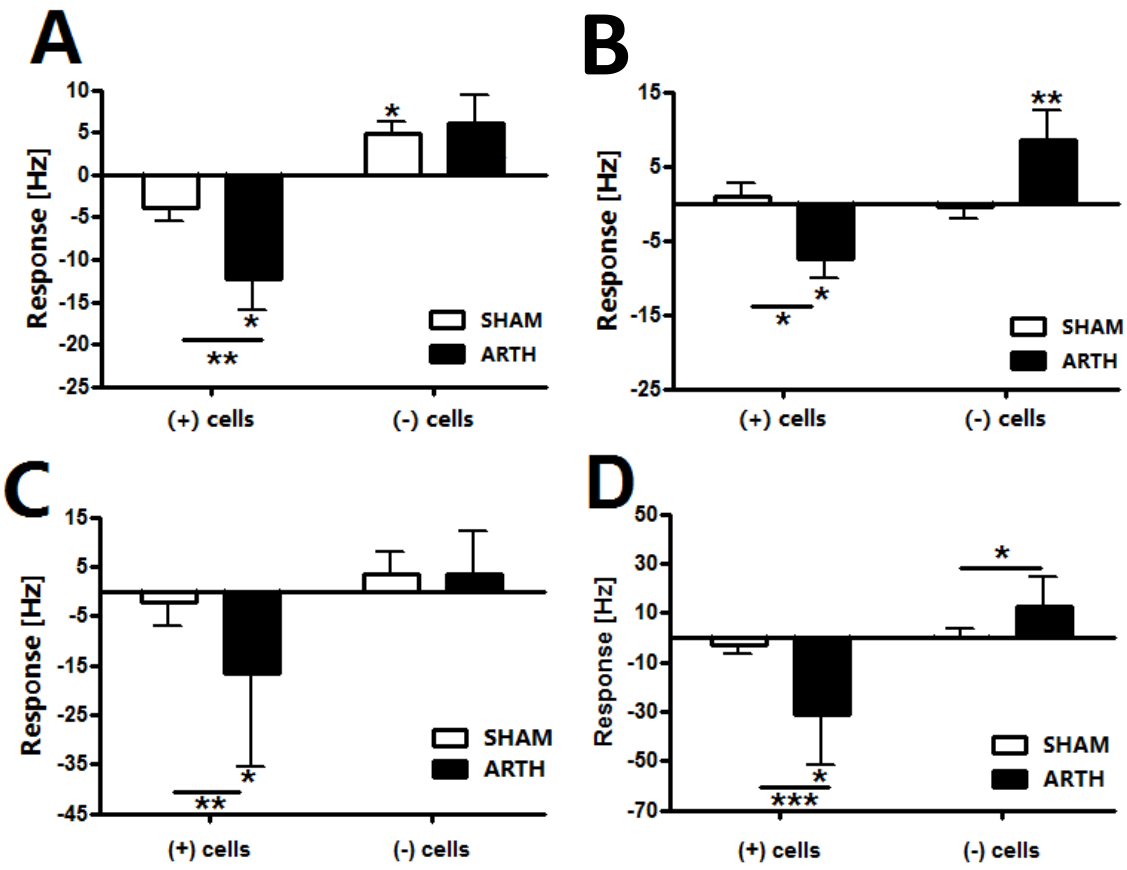

Figure 6 – Modulatory effect of PVN pharmacological manipulation upon evoked activity of CVLM (+) and (-) neurones in SHAM and ARTH animals after several modalities of noxious stimulation. The bars represent differences between noxious-evoked activity and baseline spontaneous activity (which is represented as the zero line in the x axis), comparisons within groups were performed using t-test analysis. Two-way ANOVA analysis was used to compare the overall effects of glutamate microinjection in the PVN between experimental groups. Note that the activity of (+) cells in ARTH animals was consistently depressed when compared with basal activity and with SHAM. (A – noxious heat stimulation to hind-paw; B – noxious mechanical stimulation of the tail; C – Noxious visceral stimulation; D – noxious cold stimulation of the hind-paw) (*p<0.05, **p<0.01, ***p<0.001)

Table 1 – Number of nociceptive-responsive CVLM neurones, before and after the induction of ARTH, according to their response to heat/pinch/CRD/cold noxious stimulation. (The number of cells found in ARTH animals is shown in bold) (C – controls; A – Arthritic; CRD – colorectal distension).

Response to Heat			Response to Pinch			Response to CRD			Response to Cold		
	C	**A**		C	**A**		C	**A**		C	**A**
(+) cells	44	**48**	(+) cells	21	**18**	(+) cells	9	**12**	(+) cells	14	**15**
			(-) cells	9	**11**	(-) cells	11	**13**	(-) cells	13	**23**
			(=) cells	14	**19**	(=) cells	24	**23**	(=) cells	17	**10**
(-) cells	17	**19**	(+) cells	9	**11**	(+) cells	6	**2**	(+) cells	2	**2**
			(-) cells	6	**4**	(-) cells	8	**7**	(-) cells	13	**10**
			(=) cells	2	**4**	(=) cells	3	**10**	(=) cells	2	**7**
(=) cells	17	**12**	(+) cells	6	**2**	(+) cells	3	**0**	(+) cells	4	**3**
			(-) cells	0	**4**	(-) cells	2	**3**	(-) cells	4	**7**
			(=) cells	12	**6**	(=) cells	12	**9**	(=) cells	9	**2**

Chapter 2.3.3

Pinto-Ribeiro F, Monteiro AM, Amorim D, Pertovaara A, Almeida A.

Descending pronociception from the dorsomedial nucleus of the hypothalamus (DMH) in acute pain is mediated by the rostral ventromedial medulla (RVM) but is absent in arthritic animals

(Manuscript in preparation)

Descending pronociception from the dorsomedial nucleus of the hypothalamus (DMH) in acute pain is mediated by the rostral ventromedial medulla (RVM) but is absent in arthritic animals

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Keywords: Dorsomedial hypothalamic nucleus; Arthritic pain; Descending pain modulation; Hypothalamus; Pronociception

Abbreviations:

DMH – Dorsomedial nucleus of the hypothalamus;

RVM – rostral ventromedial medulla

PAG – periaductal gray matter

CRTL – control animals/controls

ARTH – arthritic animals/arthritis

GLU – glutamate

LIDO – lidocaine

NPS – noxious peripheral stimulation

HPA – hypothalamus-pituitary-adrenals axis

Abstract

The dorsalmedial nucleus of the hypothalamus (DMH) has recently been implicated in stress-induced hyperalgesia, but the mechanisms underlying forebrain-mediated facilitation of pain are still poorly understood. Moreover, due to its involvement in the mediation of the stress response, the DMH might play an important role in the hyperalgesia of chronic inflammatory diseases, as these pathologies are known to evoke the stress response. The role of the DMH upon pain modulation in NAIVE, SHAM and arthritic (ARTH) rats was studied. Intracerebral administration of glutamate (*vs.* saline) in the DMH of NAIVE and SHAM decreased noxious-evoked thresholds in both the tail-flick and paw-withdrawal tests, whereas lidocaine block of the DMH activity had an opposite effect, increasing nociceptive thresholds. These data confirmed the existence of a descending facilitatory drive from the DMH. Surprisingly, glutamate reduction of DMH pain-like facilitation was absent in ARTH animals. To further evaluate the mechanisms underlying this phenomenon, an electrophysiological analysis of the effect of the DMH upon the activity of pain modulatory cells in the RVM was performed in SHAM and ARTH rats undergoing noxious-evoked peripheral stimulation. In SHAM animals, DMH-induced hyperalgesia is, at least in part, tonically mediated by RVM cells since lidocaince in the DMH inhibited and facilitated the activity of presumptive pronociceptive ON-cells and antinociceptive OFF-cells, respectively. Similarly to what was observed in noxious-evoked pain-like behavioural tests, DMH descending drive upon RVM nociceptive cells was absent in ARTH animals. These data point to a role of DMH nociceptive facilitation in acute behaviour defensive responses to noxious stimuli and in the enhancement of spontaneous, but not noxious-evoked, responses in chronic (arthritic) pain.

1. Introduction

The dorsomedial nucleus of the hypothalamus (DMH) is implicated in a variety of physiological responses including feeding (Chen *et al.*, 2008), thermoregulation (Dimicco and Zaretsky, 2007), cardiovascular function (DiMicco *et al.*, 1986), autonomic responses to acute stress (DiMicco *et al.*, 2002), anxiety (Shekhar, 1993) and modulation of the stress axis activity (Boudaba *et al.*, 1997). Activation of GABA receptors in the DMH have been shown to inhibit the autonomic aspects of the response to stress and anxiety in both anesthetized (DiMicco and Monroe, 1998) and wake behaving animals (Stotz-Potter *et al.*, 1996). Anatomical studies have demonstrated that the majority of DMH afferents arise from other nuclei within the hypothalamus, although there are a few significant projections from the telencephalon and brainstem (Thompson *et al.*, 1996). Conversely, DMH efferent projections target not only intrahypothalamic areas, but also the thalamus, the telencephalon and the brainstem (Thompson and Swanson, 1998). More recently, as part of the network involved in the activation of the stress response, a novel role for the DMH in descending pain modulation has been proposed. It has been shown that the DMH contributes to stress-induced hyperalgesia through direct and indirect connections to the raphe nuclei (Samuels *et al.*, 2004; Sarkar *et al.*, 2007) in the rostral ventromedial medulla (RVM), an area implicated in supraspinal pain control (Almeida et al., 2006; Heinricher *et al.*, 2009). Additionally, disinhibition of the DMH induced activation of RVM pronociceptive ON-cells and suppression of RVM antinociceptive OFF-cell firing, which is in accordance with behavioural hyperalgesia (Martenson *et al.*, 2009).

In terms of pain perception, hyperalgesia or the increased perception of pain after a noxious stimulus can be associated either to acute or chronic pain (Millan, 1999). In inflammatory diseases, such as arthritis, the initial peripheral sensitization of nociceptors by injury (McMahon *et al.*, 2008) cause primary hyperalgesia which, as the area affected enlarges, results in secondary hyperalgesia (Basbaum *et al.*, 2009). Additionally, increased peripheral nociceptive inputs upon spinal dorsal horn neurons sensitize the ascending tracts causing central sensitization (Dubner, 2005; Woolf and Salter, 2006), which if sustained in time alters the normal functioning of the brain and its descending modulatory pain pathways (Burgess *et al.*, 2002). During the process of pain chronification, both facilitatory and inhibitory circuits are activated (Herrero and Cervero., 1996; Gozariu *et al.*, 1998) and dynamic temporal changes in synaptic activation in the brainstem are observed. Accordingly, the activity of both ON- and OFF-

cell activity increased in arthritis (Miki *et al.*, 2002), although the net effect of the RVM descending modulation has not yet been established.

Our purpose in this study was to: (i) evaluate the effect of pharmacological manipulation of the DMH upon the behavioural responses of rats to noxious peripheral stimulation; (ii) associate the behavioural data with possible changes in the electrophysiological activity of RVM nociceptive ON- and OFF-neurones; (iii) study the involvement of the DMH in the hyperalgesia observed in animals with monoarthritis.

2. Experimental procedures

2.1. Animals, anaesthesia and ethical issues

The experiments were performed in adult male Wistar Han rats with 175–250g (Charles Rivers, Barcelona, Spain). The experimental protocol was approved by the Institutional Ethical Commission and followed the European Community Council Directive 86/609/EEC for the use of experimental animals. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

For the experimental surgery and electrophysiological sessions, anaesthesia was induced by administering pentobarbitone (50 mg/kg, i.p.) and maintained by infusing pentobarbitone (15–20 mg/kg/h, i.p.) when necessary. The level of anaesthesia was frequently monitored by observing the size of the pupils, the general muscle tone and behavioural responses to noxious pinching. Importantly, the anaesthesia level was maintained in an identical fashion when studying control and arthritic animals to avoid a potential influence of this factor amongst groups. A warming blanket was used to maintain the body temperature within physiological range. At the completion of the experiment, animals received a lethal dose of pentobarbitone.

2.2. Recording of neuronal responses in the rostral ventromedial medulla (RVM)

In order to perform electrophysiological recordings of the activity of RVM neurones after being anaesthetized (as described above), the animals were placed in a standard stereotaxic frame. The skull was exposed and a hole drilled for placement of a recording electrode in the RVM (5.88 mm posterior to interaural, 0.4–0.6 mm lateral from the midline, and 8.8 mm ventral from the dura mater) according to the atlas of Paxinos and Watson (2005). Single neurone activity was recorded extracellularly with lacquer-coated tungsten electrodes (tip impedance 3–10 MΩ at 1

kHz) and then amplified and filtered using standard techniques. Data sampling was performed with a computer connected to a CED Micro 1401 interface and using Spike 2 software (Cambridge Electronic Design, Cambridge, U.K.).

Recording of RVM neurones was started after the animal was under light anaesthesia; i.e., the animals gave a brief withdrawal response to noxious pinch, but the pinch did not produce any longer lasting motor activity, nor did the animals have spontaneous limb movements. RVM neurones were classified based on their response to noxious heating (54 °C) of the tail with a tail-flick device (Ugo Basile, Comerio, Italy). The functional classification of RVM neurones followed the scheme developed earlier by Fields and colleagues (1983).

Briefly, neurones displaying an increase in firing rate evoked by heat stimulation of the tail, associated with its withdrawal, were considered ON-cells, while those decreasing its activity were classified as OFF-cells **(Fig. 1)**. Neurons displaying no or only a negligible (< 10%) change in their discharge rates as a response to noxious stimulation were considered NEUTRAL-cells and were not studied further in this investigation. If a neuron could not be classified it was excluded from the study.

Characterization of the response properties of RVM cells consisted of the following assessments performed successively: (i) Spontaneous activity; (ii) Response to heating of the tail, and (iii) Response to pinching of the tail for 5 s by a surgical clamp that produced painful sensation when applied to the hand of the experimenter.

When analyzing responses of RVM neurons to peripheral stimulation, the baseline discharge frequency recorded during a corresponding period just before the stimulation was subtracted from the discharge frequencies determined during stimulation; i.e., positive values represent excitatory responses evoked by peripheral stimulation and negative ones inhibitory responses.

The animals used in recordings had a guide cannula for drug administration into the DMH (see section 2.3) (**Fig. 2**). Electrophysiological experiments were performed one to two weeks after fixation of the guide cannula to the skull, as described below. After determining the responses of a RVM neurone to peripheral stimulation, the phasic modulation of the discharge rate of RVM neurones by exciting DMH neurones was assessed by microinjecting glutamate (GLU, 50 nmol in 0.5 μL) into the DMH using methods described above. The discharge rate of the RVM cells was followed up to 5 min after the injection of GLU. Thereafter, tonic control of the RVM by the DMH

was assessed by blocking the activity of the nucleus by microinjecting lidocaine (LIDO, 4% in 0.5 μL) into the DMH and following the discharge rate of RVM neurones up to 30 min. All results from the drug administration were plotted against the values obtained for the same time points after saline (SAL) injection in the DMH.

2.3. Procedures for intracerebral microinjections

For intracerebral drug administration, the rats were placed in a stereotaxic frame and a stainless steel guide cannula (26 gauge; Plastics One, Roanoke, VA) was implanted in the brain according to the coordinates of the atlas by Paxinos and Watson (1998). The tip of the guide cannula was positioned 1 mm above the desired injection site in the DMH (RC, 5.76 mm interaural; LM, 0.4 mm; DV, 8.5 mm to the interaural line) **(Figs. 1)**. After the guide cannula was fixed into the skull using a dental screw and dental cement, a dummy cannula was inserted into the guide cannula and the top was closed. Animals were allowed to recover from surgery for one week before testing.

Test-drugs were administered in the DMH through a 33-gauge injection cannula (Plastics One, Bilaney, Germany) inserted into and protruding 1 mm beyond the tip of the guide cannula. The microinjection was made using a 1.0-μL-Hamilton syringe connected to the injection cannula by a polyethylene catheter (PE-10; Plastics One, Bilaney, Germany). The injection volume was 0.5 μL and therefore, the spread of the injected drugs within the brain was expected to be 1 mm (Myers, 1966). The efficacy of injection was monitored by watching the movement of a small air bubble through the tubing. The injection lasted 20s and the injection cannula was left in place for an additional 30s to minimize the return of drug solution back to the injection cannula. Brain injection sites were histologically verified from post-mortem sections and plotted on standardized sections derived from the stereotaxic atlas of Paxinos and Watson (2005).

2.4. Induction of arthritis

The induction of arthritis (ARTH) was performed 21 days before the actual experiments, as described in detail elsewhere (Ansah and Pertovaara, 2007). Briefly, 3% kaolin and 3% carrageenan (Sigma, St. Louis, MO, USA) were dissolved in distilled water and injected into the synovial cavity of the left knee joint at a volume of 0.1 mL. This model produces mechanical hyperalgesia, which begins just in a few hours after surgery and extends up to 8 weeks (Radhakrishnan *et al.*, 2003). In each animal, development of arthritis was verified 1–2 h prior to

each experiment. Only those rats that vocalized every time after five flexion–extension movements of the knee joint were considered to have arthritis, and they were included in the arthritis group. An experimental group used as SHAM animals were also injected with 0.1 mL saline in the synovial cavity of the left knee joint. SHAM animals did not vocalize to any of the five consecutive flexion–extension movements of the knee joint.

2.5. Behavioural assessment of nociception

The rats were habituated to the experimental conditions by allowing them to spend 1–2 h daily in the laboratory during two to three days preceding any testing. For assessing nociception in unanaesthetized animals, radiant heat-induced latency of the tail (Tail-flick test, device model 37360, Ugo Basile, Comerio, Italy) and hindpaw (Hargreaves test, device model, Ugo Basile, Comerio, Italy) was determined using radiant heat equipment. In each drug treatment session, the withdrawal latency was assessed prior to drug treatment and at various intervals following the intracerebral injections. At each time point, the measurement was repeated twice at an interval of 1 min and the mean of these values was used in further calculations. Cut-off time was 15s.

2.6. Drugs

Solutions for intracerebral drug administration in the DMH were prepared with sterilized saline 0,9% (Unither, Amiens, France; pH 7,2) except for lidocaine, which was acquired as a solution. Each injection volume, 0.5 µL, contained either (1) 50 nmol of glutamate (Merck, Darmstadt, Germany) or lidocaine 2 % (B. Braun Medical, Portugal). These doses were chosen according to previous studies (Pinto-Ribeiro, 2008). Control injections were performed with saline (SAL) and we used them as base of comparison to the drugs, in order to avoid any confounding effect that might result from the injection itself.

2.7. Animal groups

For the purpose of this study the animals were divided in three groups NAIVE, SHAM and ARTH. In the first set of experiments, NAIVE animals were used to test DMH facilitatory action while in a second set, SHAM and ARTH animals were used to evaluate its role in a chronic inflammatory context. In both experimental sets, all the animals were microinjected with saline (SAL), glutamate (GLU) and lidocaine (LIDO) every two days.

2.8. Course of the behavioural study

One to two weeks following induction of the arthritis and at least one week following insertion of the guide cannula for DMH injections, the efficacy of DMH-induced phasic and tonic modulation of spinal nociception was determined by assessing the effect of glutamate and lidocaine injection in the DMH upon the heat-evoked spinal withdrawal reflex in unanesthetized ARTH and NAIVE and SHAM animals. Physiological saline was used as a control for the volume of injection (in NAIVE, SHAM and ARTH groups) while NAIVE and SHAM animals were used as controls to ARTH animals. In these experiments, the latency of the withdrawal response was assessed 30 s, 5 min, 15 min and 30 min following the injection **(Fig. 3)**. The latency measured 30 s after glutamate injection and 15 min after lidocaine injection was used in further calculations, since the maximum effects of the studied compounds are obtained at these time points. The interval between behavioural assessments of glutamate-, lidocaine- or saline-induced effects was at least two days and the order of testing different compounds varied between the animals.

2.9. Course of the electrophysiological study

Electrophysiological recordings of RVM neurones were performed under pentobarbitone anesthesia in different animals one to two weeks following the induction of arthritis and at least one week following the insertion of the guide cannula for DMH injections. In RVM recordings, the response properties of the neurons were assessed by determining spontaneous activity and the response to noxious heating of the tail and tail pinch. Then, the change in spontaneous activity of RVM neurones following successive microinjections of glutamate and lidocaine at a 30 min interval into the DMH was assessed as described in detail above **(Fig. 3)**. Search for the next neuron to be studied started about 30 min after the testing of the previous one was completed. At the end of the recording session, electrolytic lesions were made in the recording sites, the animals were given a lethal dose of pentobarbitone and the brains were removed for histological verification of the recording and injection sites.

2.10. Statistics

The differences in spontaneous activity between controls and arthritic animals were tested using a t-test comparison. The ANOVA repeated measures test was used while analysing the effectiveness of the administration of a compound in a group of cells throughout time (**Fig. 6**). The statistical assessment of differences in neuronal activity between experimental groups during the pharmacological treatments was assessed using two-way analysis of variance (ANOVA)

followed by Bonferroni *post-hoc* test (**Figs. 7-9**). A value of $p<0.05$ was considered to represent a significant difference.

3. Results

3.1 The induction of monoarthritis did not alter the behaviour in nociceptive tests.

In order to assess if monoarthritis, induced in the knee joint of right hind-paw, altered by itself baseline withdrawal latencies we compared the PF latencies amongst the left and right side and between groups. Baseline PF latencies for NAIVE, SHAM and ARTH animals were identical amongst the left and the right paws (**Table 1**) and between groups [ANOVA1W, $F_{(2,228)}=1,114$, $p=0,4132$].

3.2 The behavioural pronociceptive drive from the DMH is lost in monoarthritis

To investigate a possible phasic role of the DMH in pain modulation in monoarthritis, tail-flick (TF) and paw-flick (PF) latencies were assessed after GLU microinjection in this nucleus in NAIVE, SHAM and ARTH animals. Overall, DMH activation by GLU microinjection significantly decreased TF latency in NAIVE and SHAM animals but not in ARTH animals. Activation of the DMH by GLU significantly decreased TF latencies in NAIVE and SHAM animals when compared to SAL in NAIVE and SHAM [$ANOVA_{2w}$, $F_{(1,56)}=33,37$, $p<0.0001$; Bonferroni, $NAIVE_{SAL}$ x $NAIVE_{GLU}$, $p<0.001$ and $SHAM_{SAL}$ x $SHAM_{GLU}$, $p<0.01$]. Additionally, GLU in the DMH decreased TF latencies in NAIVE and SHAM when compared to ARTH [$ANOVA_{2w}$, $F_{(2,56)}=4,915$, $p=0,0108$; Bonferroni, $NAIVE_{GLU}$ x $ARTH_{GLU}$, $p<0.001$ and $SHAM_{GLU}$ x $ARTH_{GLU}$, $p<0.05$] (**Fig. 4A**). Note that in ARTH animals no difference was observed between SAL and GLU administration to the DMH (**Fig. 4A**).

Identical results were obtained in the PF test, DMH activation by GLU microinjection significantly decreased PF latencies in NAIVE and SHAM animals but not in ARTH animals. PF latencies of NAIVE and SHAM animals, after GLU microinjection in the DMH, were significantly decreased when compared to SAL in NAIVE and SHAM [$ANOVA_{2w}$, $F_{(1,59)}=14.77$, $p=0.003$; Bonferroni, $NAIVE_{SAL}$ x $NAIVE_{GLU}$, $p<0.001$ and $SHAM_{SAL}$ x $SHAM_{GLU}$, $p<0.05$]. Additionally, GLU in the DMH decreased PF latencies in NAIVE and SHAM when compared to ARTH animals [$ANOVA_{2w}$, $F_{(2,59)}=6.045$, $p=0,0041$; Bonferroni, $NAIVE_{GLU}$ x $ARTH_{GLU}$, $p<0.01$ and $SHAM_{GLU}$ x $ARTH_{GLU}$, $p<0.05$] (**Fig. 4B**). Note

that again the PF latencies of the ARTH group were not altered by the activation of the DMH by GLU (**Fig. 4B**).

On the other hand, to evaluate a possible tonic role of the DMH in pain modulation in monoarthritis, TF and PF latencies were assessed after LIDO microinjection in this nucleus in NAIVE, SHAM and ARTH animals. Overall, DMH inhibition by LIDO microinjection significantly increased TF and PF latencies in NAIVE and SHAM animals but not in ARTH animals. In the TF test, a decrease in pain-like behaviour after LIDO administration in the DMH was observed, in NAIVE and SHAM animals, after 10 [$ANOVA_{2w}$, $F_{(3,155)}=10.27$, $p<0.0001$; SAL_{10} x $LIDO\text{-}NAIVE_{10}$, $p<0.01$ and SAL_{10} x $LIDO\text{-}SHAM_{10}$, $p<0.01$] and 20 minutes [$F_{(3,155)}=10.27$, $p<0.0001$; SAL_{20} x $LIDO\text{-}NAIVE_{20}$, $p<0.01$ and SAL_{20} x $LIDO\text{-}SHAM_{20}$, $p<0.01$] of injection (**Fig. 5A**) but not in ARTH animals. Additionally, at 10 minutes, the latencies of both NAIVE [$F_{(2,155)}=3.281$, $p=0.04$; LIDO ($NAIVE_{10}$ x $ARTH_{10}$), $p<0.05$] and SHAM [$F_{(2,155)}=3.281$, $p=0.04$; LIDO ($SHAM_{10}$ x $ARTH_{10}$), $p<0.05$] were significantly increased when compared to ARTH (**Fig. 5A**). Note that in ARTH animals no difference was observed between SAL and GLU administration to the DMH (**Fig. 5A**).

Similar results were obtained in the PF test as withdrawal latencies of NAIVE and SHAM animals after LIDO injection in the DMH were significantly increased when compared to SAL in NAIVE and SHAM at 10 minutes [$ANOVA_{2w}$, $F_{(3,117)}=8.984$, $p<0.0001$; SAL_{10} x $LIDO\text{-}NAIVE_{10}$, $p<0.001$ and SAL_{10} x $LIDO\text{-}SHAM_{10}$, $p<0.001$] and 20 minutes [$ANOVA_{2w}$, $F_{(3,117)}=8.984$, $p<0.0001$; SAL_{20} x $LIDO\text{-}NAIVE_{20}$, $p<0.001$ and SAL_{20} x $LIDO\text{-}SHAM_{20}$, $p<0.01$]. Again, TF latencies were not altered in ARTH-treated animals (**Fig. 5B**). Withdrawal latencies of NAIVE [$ANOVA_{2w}$, $F_{(2,117)}=3.245$, $p<0.043$; LIDO ($NAIVE_{10}$ x $ARTH_{10}$), $p<0.05$] and SHAM [$ANOVA_{2w}$, $F_{(2,117)}=3.245$, $p<0.043$; LIDO ($SHAM_{20}$ x $ARTH_{20}$), $p<0.05$] animals were significantly higher than ARTH 20 minutes after LIDO administration (**Fig. 5B**). Note that once more the PF latencies of the ARTH group were not altered by the activation of the DMH by LIDO (**Fig. 5B**).

3.3 Downstream nociceptive modulation from the DMH is partially mediated by the RVM

To investigate a possible downstream relay of the pronociceptive phasic and tonic drive from the DMH, the effect of its pharmacological manipulation, activation by glutamate or inhibition through lidocaine microinjection, upon the activity of RVM cells was analysed during peripheral thermal (TS) and mechanical (MS) noxious stimulation.

3.3.1. Spontaneous activity

A total of 102 cells of SHAM animals [69 ON-cells - 39 ON-cells to mechanical stimulation (MS) and 30 ON-cells to thermal stimulation (TS) - and 33 OFF-cells - 19 OFF-cells to MS and 14 OFF-cells to TS] and 253 cells of ARTH animals [137 ON-cells - 71 ON-cells to MS and 66 ON-cells to TS - and 116 OFF-cells - 65 OFF-cells to MS and 51 OFF-cells to TS] were analyzed in the RVM. Since no difference was observed in spontaneous activity [ON-cells, ANOVA2W, F(1,86)=1.031, p=0.32 and OFF-cells, ANOVA2W, F(1,10)=0.035, p=0.85] between NAIVE and SHAM animals, the results presented within the electrophysiological section correspond to comparisons between ARTH and SHAM animals.

Microinjection of SAL in the DMH had no effect upon the spontaneous discharge rate of the RVM cells in either SHAM [ON-cells, $ANOVA_{rm}$, $F_{(2,47)}$=0.57, p=0.57 and OFF-cells, $ANOVA_{rm}$, $F_{(2,14)}$=1.07, p=0.39] or ARTH [ON-cells, $ANOVA_{rm}$, $F_{(2,95)}$=1.055, p=0.8 and OFF-cells, $ANOVA_{rm}$, $F_{(2,95)}$=0.027, p=0.99] (**Fig. 6**). However, the spontaneous activity of RVM cells before intracerebral DMH drug administration was significantly decreased in ARTH (ON-cell, 0.30±1.22 spikes/s and OFF-cell, 2.49±0.31 spikes/s) when compared to SHAM (ON-cell, 3.25±4.88 spikes/s and OFF-cell, 8.17±7.44 spikes/s), for both ON-cells [$ANOVA_{2w}$, $F_{(1,126)}$=26.22, p<0.0001; $SHAM_{PI}$ x $ARTH_{PI}$ p<0.01, $SHAM_{I}$ x $ARTH_{I}$ p<0.01 and $SHAM_{PTI}$ x $ARTH_{PTI}$ p<0.05] (**Fig. 6A**) and OFF-cells [$ANOVA_{2w}$, SHAM x ARTH, $F_{(1,72)}$=42.63, p<0.001; Bonferroni, $SHAM_{PI}$ x $ARTH_{PI}$ p<0.01, $SHAM_{I}$ x $ARTH_{I}$ p<0.001 and $SHAM_{Po-I}$ x $ARTH_{Po-I}$ p<0.001] (**Fig. 6B**).

In SHAM animals, DMH activation by GLU significantly increased ON-cell [$ANOVA_{rm}$, $F_{(2,53)}$=4.15, p=0.02; Bonferroni, Glu_{PI} x Glu_{Po-I}, p<0.05] (**Fig. 7A**) and decreased OFF-cell [$ANOVA_{rm}$, $F_{(2,7)}$=4.45, p=0.04; Bonferroni, Glu_{PI} x Glu_{Po-I}, p<0.05] (**Fig. 7B**) spontaneous activity. This effect is absent in the ARTH group where GLU administration did not alter the spontaneous activity of RVM ON- [$ANOVA_{rm}$, $F_{(2,80)}$=1.25, p=0.29] and OFF-cells [$ANOVA_{rm}$, $F_{(2,44)}$=1.21, p=0.31]. The inhibition of the DMH by LIDO, in SHAM animals, had no effect upon the spontaneous activity of both RVM ON-cells [$ANOVA_{rm}$, $F_{(5,41)}$=2.05, p=0.10] (**Fig. 7C**) and OFF-cells [$ANOVA_{rm}$, $F_{(5,53)}$=0.66, p=0.65] (**Fig. 7D**) while in ARTH animals, spontaneous ON-cell activity was decreased [$ANOVA_{rm}$, $F_{(5,71)}$=3.10, p<0.03; Bonferroni, $Lido_{PI}$ x $Lido_{10}$ p<0.05] (**Fig. 7C**) but no effect was observed in OFF-cells activity [$ANOVA_{rm}$, $F_{(5,113)}$= 1.30, p=0.27] (**Fig. 7D**).

3.3.2 The pronociceptive action of the DMH is partially modulated by RVM cell activity but is lost in monoarthritis

In SHAM animals, DMH activation by GLU significantly increased ON-cell heat-evoked [$ANOVA_{2W}$, $F_{(1,54)}$=31.56, p<0.0001; Bonferroni, Glu_{PI} x Glu_{I}, p<0.05] (**Fig. 8A**) and mechanical-evoked [$ANOVA_{2W}$, $F_{(1,74)}$=18.9, p=0.02; Bonferroni, Glu_{PI} x Glu_{I}, p<0.05] noxious activity (**Fig. 8B**) and also increased OFF-cell heat-evoked [$ANOVA_{2W}$, $F_{(1,42)}$=5.75, p=0.02; Bonferroni, Glu_{PI} x Glu_{I}, p<0.05] (**Fig. 8C**) and mechanical-evoked [$ANOVA_{2W}$, $F_{(1,52)}$=18.7, p<0.0001; Bonferroni, Glu_{PI} x Glu_{I}, p<0.05] noxious activity (**Fig. 8D**). This effect is absent in the ARTH group, where GLU administration did not alter the noxious-evoked activity of both RVM ON- and OFF-neurones.

In accordance to a facilitatory role for the DMH, its inhibition by LIDO significantly decreased ON-cell heat-evoked at 20 minutes [$ANOVA_{2W}$, $F_{(1,110)}$=2.84, p=0.03; Bonferroni, $LIDO_{PI}$ x $LIDO_{20}$, p<0.05] (**Fig. 9A**) and mechanical-evoked activity at 10, 20 and 30 minutes [$ANOVA_{2W}$, $F_{(1,144)}$=11.06, p<0.0001; Bonferroni, $LIDO_{PI}$ x $LIDO_{10}$, p<0.001; $LIDO_{PI}$ x $LIDO_{20}$, p<0.001; $LIDO_{PI}$ x $LIDO_{30}$, p<0.001] (**Fig. 9B**), while it increased OFF-cell heat-evoked at 10 and 20 minutes [$ANOVA_{r2W}$, $F_{(1,60)}$=14.95, p=0.003; Bonferroni, $LIDO_{PI}$ x $LIDO_{10}$, p<0.01 and $LIDO_{PI}$ x $LIDO_{20}$, p<0.05] (**Fig. 9C**) and mechanical-evoked activity at 10 and 20 minutes [$ANOVA_{2W}$, $F_{(1,60)}$=14.95, p=0.003; Bonferroni, $LIDO_{PI}$ x $LIDO_{10}$, p<0.01 and $LIDO_{PI}$ x $LIDO_{20}$, p<0.05] (**Fig. 9D**). This effect is absent in the ARTH group, where LIDO administration did not alter the noxious-evoked activity of both RVM ON- and OFF-cells.

4. Discussion

In this work we demonstrated that the DMH has a pronociceptive (phasic and tonic) role enhancing pain-like behaviours in rodents during peripheral noxious stimulation. DMH facilitation is partly mediated by the RVM pain-modulatory ON- and OFF-cells. Unlike other supraspinal areas that enhance nociception in chronic pain, the facilitatory effect of the DMH is absent in arthritic animals.

4.1 The DMH in pain modulation

In 2009, Martensen and colleagues proposed that the DMH was involved in stress-induced hyperalgesia (SIH) as its desinhibition by gamma aminobutyric acid (GABA) antagonists resulted in behavioural hyperalgesia, an effect mediated by the RVM. Our results from the

pharmacological manipulation of this area in NAIVE and SHAM animals confirm that the DMH has the ability to enhance nociception since glutamate stimulation of the DMH decreased withdrawal latencies in nociceptive tests, whereas lidocaine block of the nucleus results in the opposite effect. Additionally, the DMH descending influence has a tonic pronociceptive role, unlike the majority of the forebrain descending influences (Gebhart, 2004) that are inhibitory. Descending facilitation, although less known, has been previously been demonstrated for other nuclei, namely the RVM (Heinricher *et al.*, 2009) and the dorsal reticular nucleus (DRt) (Lima and Almeida, 2002). Although only a few studies have focused on SIH it has been shown that the enhancement of pain perception is associated with the emotional-affective dimension of pain processing (Wang *et al.*, 2008). Humans suffering from anxiety display decreased thresholds to both noxious and innocuous stimulation when compared to non-anxious subjects (van den Hout *et al.*, 2001) and similar results were observed for rats (van Dijken *et al.*, 1992). DMH afferent projections from the amydala (AMY) (Thompson and Swanson, 1998), further support this hypothesis as the AMY is a key player in emotionality (Neugebauer *et al.*, 2003). Additionally, AMY modulation of pain was shown to be not only antinociceptive (Helmstetter *et al.*, 1998) but also pronociceptive (Ansah *et al.*, 2009). DMH-induced behavioural hyperalgesia after GLU in NAIVE and Sham animals was paralleled with an increase of ON- and a decrease of OFF-spontaneous cell activity in the RVM. Additionally, although a clear net phasic pronociceptive change in ON-/OFF-noxious evoked activity was not achieved after GLU, a tonic pronociceptive drive was reported after blocking the DMH with LIDO.

4.2 DMH and arthritis

On the other hand, the absence of an effect upon noxious-evoked withdrawal thresholds after the induction of arthritis was quite intriguing. We would expect an increase in the descending pronociceptive drive originating in the DMH since this nucleus is involved in the stress response (DiMicco *et al.*, 2002), an hyperalgesic state is present and arthritis has been shown to enhance the responsiveness of AMY neurones (Neugebauer and Li, 2003). The RVM and the DRt, as sources of possible descending facilitation, have also been shown to contribute to the maintenance of a hyperalgesic state in chronic pain (Sotgiu *et al.*, 2008; Porreca *et al.*, 2002). In clinical settings, the most common comorbities of chronic pain patients are depression and anxiety, both known to enhance pain perception (Edwards *et al.*, 2006; Ohayon and Schatzberg, 2003).

In a previous study we reported that the paraventricular nucleus of the hypothalamus (PVN) exerted a tonic inhibitory drive upon the RVM OFF-cells present only after the induction of arthritis (Pinto-Ribeiro *et al.*, 2008). In this context, the DMH efferent facilitatory drive in "intact rats" may be essential in the building of a response only to an acute challenge (fight and flight) but would not be recruited in a chronic inflammatory context. It seems possible that this apparent arthritis-induced insensibility might reflect the existence of a tonic inhibition of the DMH from other nuclei within the hypothalamus, most of which have antinociceptive roles. As the PVN shares strong projections with the DMH (Thompson *et al.*, 1996; Thompson and Swanson, 1998), we hypothesized that the PVN inhibitory influence might also target the DMH.

Since, pain is a stressor that activates the hypothalamus-pituitary-adrenal glands axis (HPA axis) of which the PVN is the main effector, a potential contribution of the DMH in arthritis could be involved in the response to physiological imbalances in order to re-establish homeostasis of the nociceptive system. In a previous study, the RVM pronociceptive drive was maintained during arthritis through a tonic enhancement of OFF-cells activity (Pinto-Ribeiro *et al.*, 2008). On the contrary, in this present study the induction of arthritis was sufficient to significantly decrease the spontaneous and noxious-evoked discharge rates of RVM cells; this discrepancy between increased vs. decreased spontaneous activity of RVM cells might be a result of a shift from facilitation to inhibition observed in descending modulatory influences during the process of pain chronification (Terayama *et al.*, 2000). Accordingly, it is important to note this specific set of experiments was performed at a later stage of the arthritis settlement (15-21 days *vs.* 7-15 days in the 2008 study), which could result in the inhibitory effect upon RVM cells. Finally, as pain pathways suffer considerable remodelling during pain chronification, it is possible that the absence of DMH facilitation could be also due to this inhibition of the RVM and to the inability of RVM neurones to respond due to the shift from facilitation to inhibition in descending modulatory influences. On the other hand, a possible role for the DMH in the development of spontaneous pain in ARTH animals was reported, as LIDO block of the DMH decreased ON-cell activity with no effect upon OFF-cells. Future studies should evaluate the potential role of the DH in the development of spontanenous pain in arthritic animals.

4.3 Conclusions

The DMH participates in pain facilitation in normal animals through descending connections with the RVM. In arthritic animals, the DMH effect is lost, at least in what concerns noxious-evoked

activity, although its involvement in spontaneous pain following arthritis remains to be elucidated. Taking into account the potential clinical relevance of the latter possibility, the role of the DMH in arthritis should be evaluated using test of on-going pain (Boyce-Rustay et al., 2010).

5. ACKNOWLEDGEMENTS

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Table 1 – Tail-flick and paw-flick baseline latencies of the NAIVE, SHAM and ARTH groups. Note that the baseline latencies were identical amongst the left and the right paws and between groups.

Tail-flick test						
	Mean Latency (sec)		**SD**		**N**	
CTRL	5,90		0,99		14	
SHAM	5,98		0,17		5	
ARTH	5,91		0,39		7	
Paw-flick test						
	Left Paw		**Right Paw**			
	Mean Latency (sec)	**SD**	**Mean Latency (sec)**	**SD**	**N**	**t-test (left-right)**
CTRL	6,21	0,84	6,44	1,16	15	$t_{(0,636)}$=0,53
SHAM	6,08	0,73	6,35	0,82	6	$t_{(0,491)}$=0,64
ARTH	6,65	1,61	6,87	1,48	9	$t_{(2,107)}$=0,07

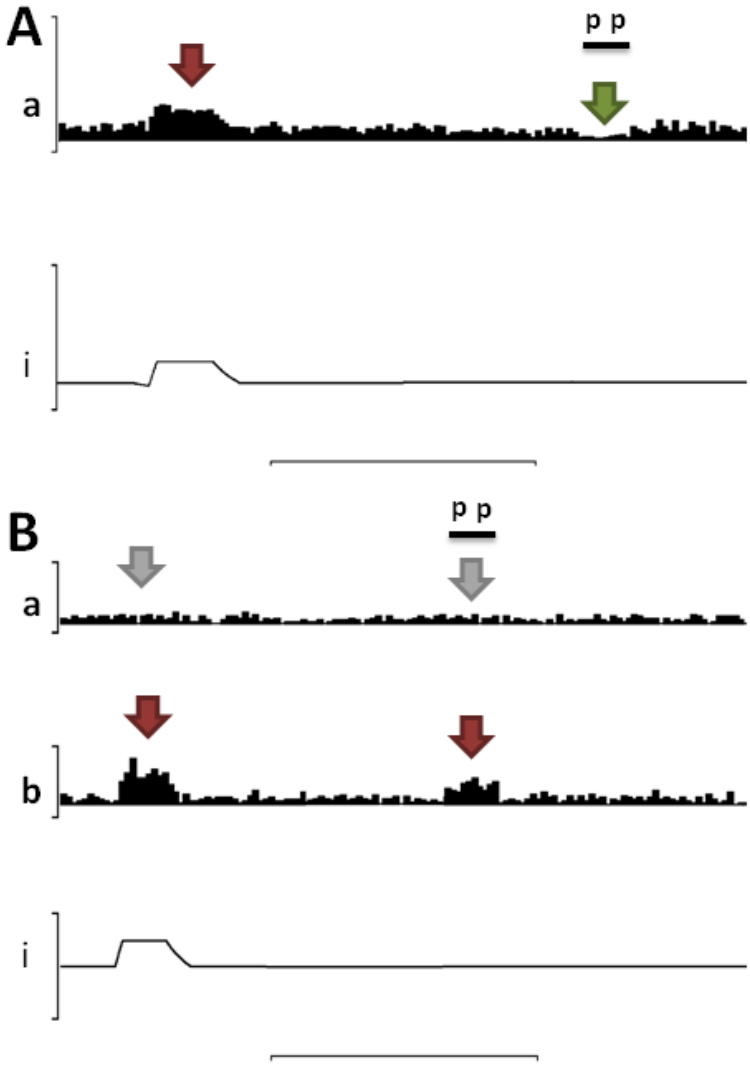

Figure 1. Examples of original recordings of the activity of RVM pain modulatory cells. A - Neurone that increases activity during noxious peripheral heat stimulation but that decreases it during mechanical noxious stimulation of the tail. B) The first neurone (a) does not respond to either modalities of peripheral noxious stimulation whereas the second neurones (b) increases its activity during both the heat and the mechanical noxious peripheral stimulation. In the graphs, *P–P* indicates the duration of the noxious tail pinch (mechanical stimulation). The vertical calibration bar for neuronal response represents 10 Hz in A and 20 Hz in B. (*red arrows* – indicate an increase in neuronal activity during noxious peripheral stimulation; *green arrow* – indicate a decrease in neuronal activity during noxious peripheral stimulation; *gray arrows* – indicate no change in neuronal activity during peripheral noxious stimulation)

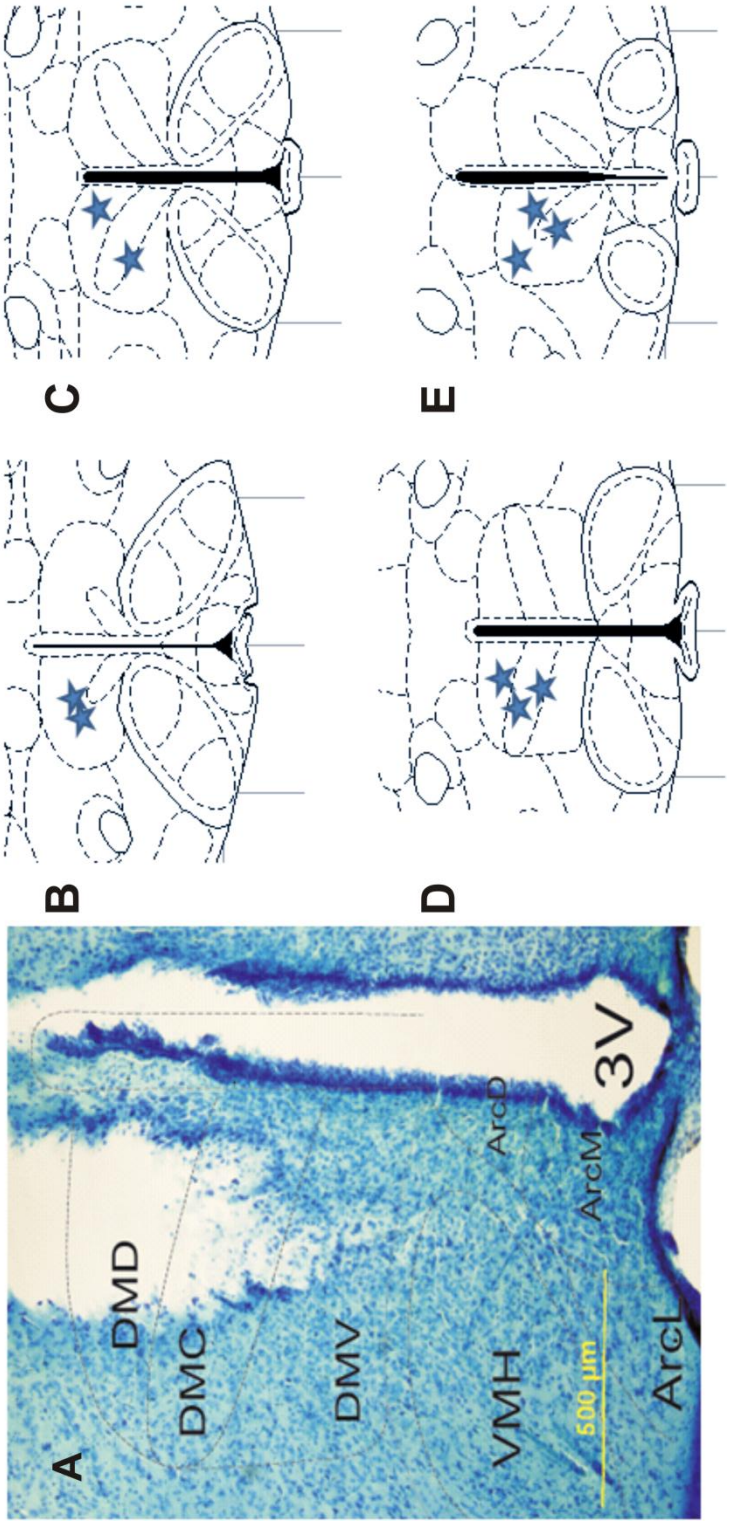

Figure 2 – Example of the drug administration site in the DMH of the rat brain and schematic representation of other injection sites. A – Injection site in the DMH (RC: -3.24 mm Bregma) (B: -3.00 mm Bregma, C: -3.12 mm Bregma, D: -3.24 mm Bregma and E: -3.36 mm Bregma).

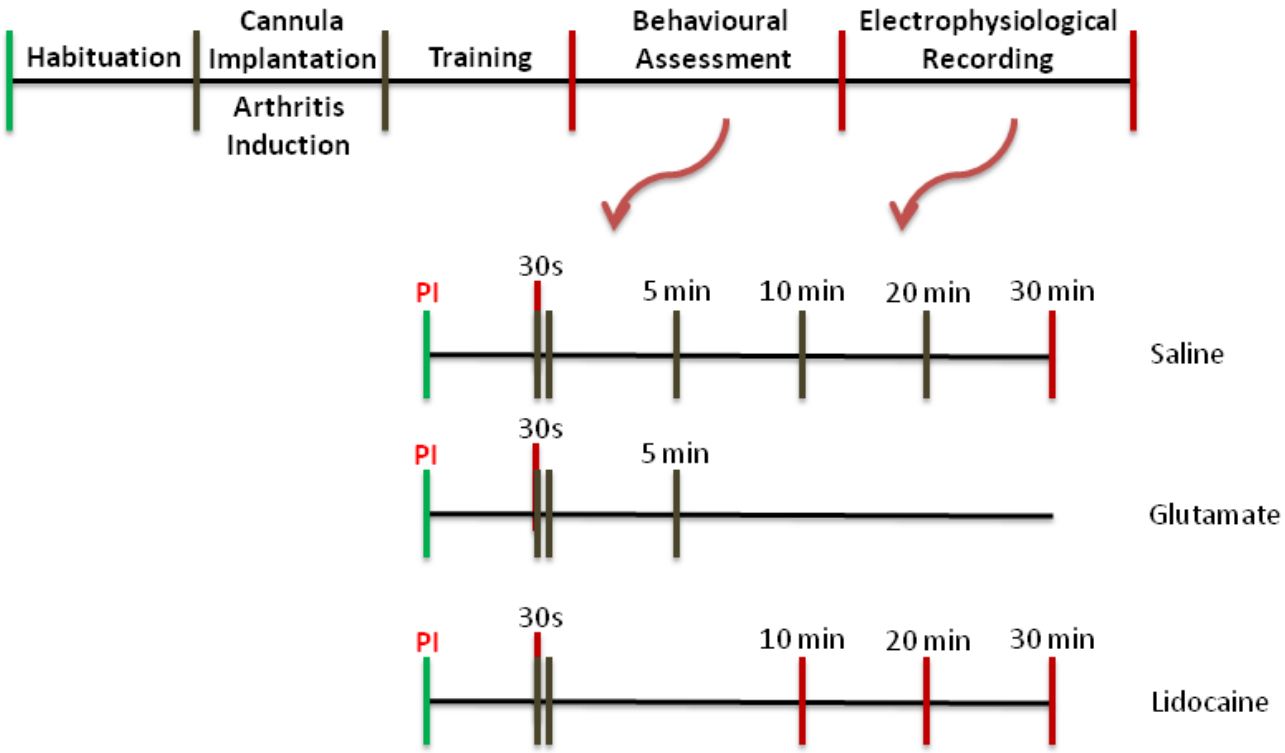

Figure 3 - Schematic representation of the experimental design. Rats used to evaluate the effect of the DMH upon descending pain modulation were accustomed to the room for 5 days after which animals were implanted with a cannula in the DMH and allowed to recover for a week. Animal belonging to the ARTH group were also injected with kaolin/carrageenan in the knee joint of the right hind paw while SHAM were injected with saline. After recovery, animals included in the behavioral study were trained in the behavioural apparatus for one week, while animals destined to the electrophysiological studies were immediately analyzed. Pharmacological tests were performed at the same points for both the behavioral and the electrophysiological studies. (PI – pre-injection; I - injection)

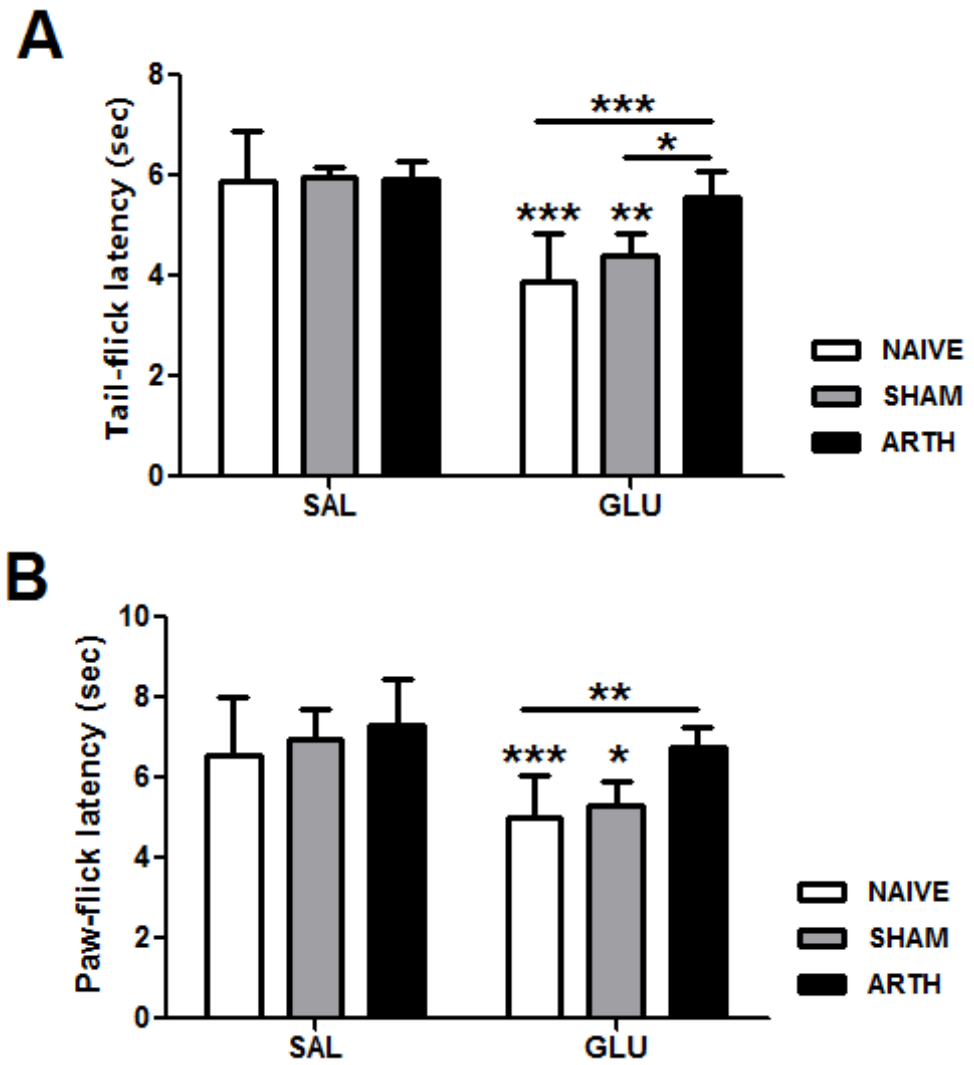

Figure 4 - Behavioural analysis of the effect of the intracerebral administration of GLU in the DMH in NAIVE(white bars), SHAM(gray bars) and ARTH(black bars) animals. The microinjection of SAL was used as a control injection (TF, n=14 and PF, n=20) for both SHAM (TF, n=5 and PF, n=6) and ARTH (TF, n=7 and PF, n=9) groups. Note that the phasic pronociceptive downstream action of the DMH is lost after the induction of ARTH as the decrease in withdrawal latency after GLU in the DMH observed in NAIVE and SHAM animals is absent in the ARTH group. (SAL – saline; GLU – glutamate; ARTH – arthritis/arthritic animals; TF – tail-flick; PW – paw-withdrawal) (Mean ± SD) *p<0.05, **p<0.01, ***p<0.001.

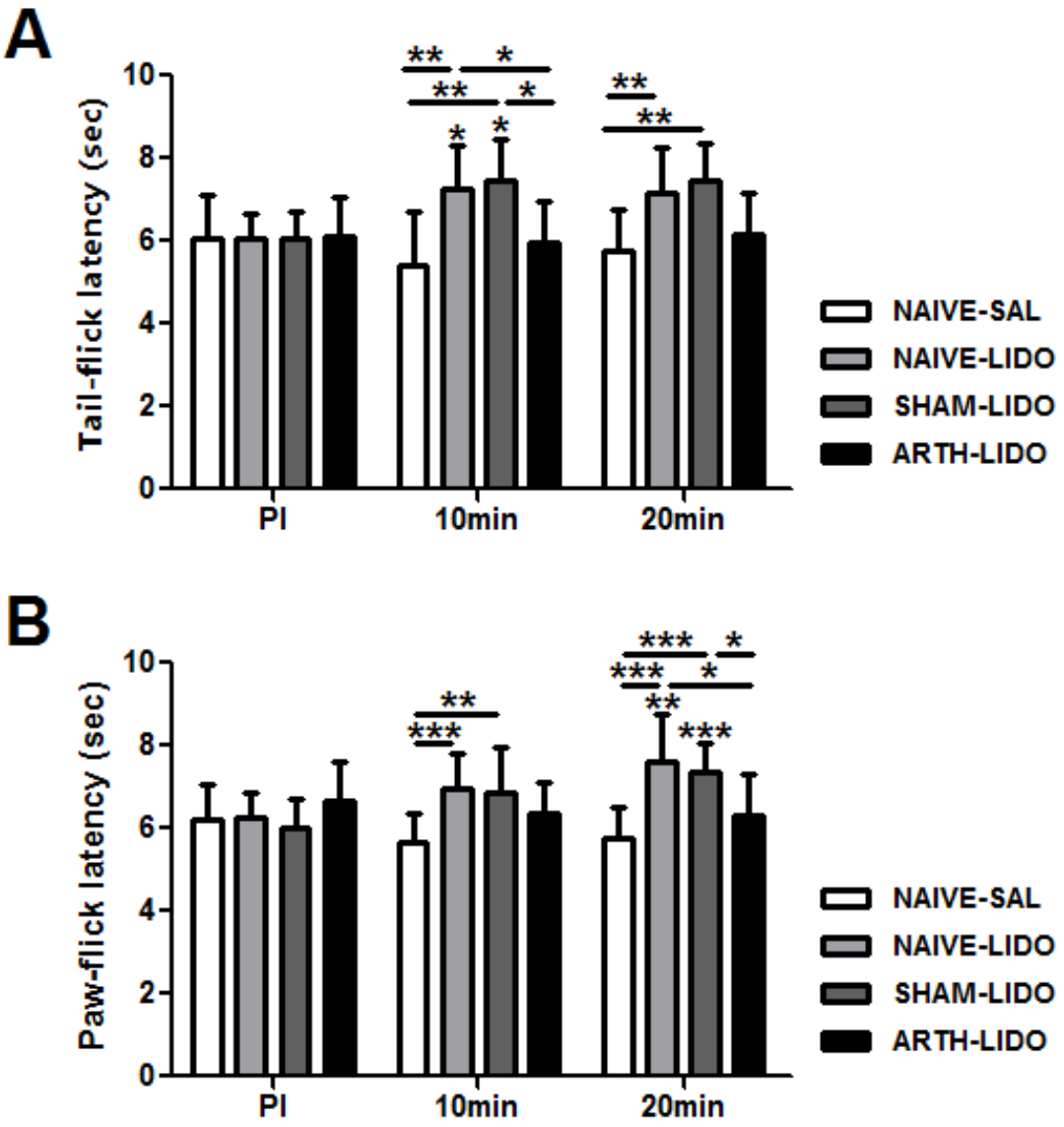

Figure 5 - Behavioural analysis of the effect of intracerebral administration of LIDO in the DMH in the tail-flick (A) and paw-flick (B) tests in NAIVE(lightgray bars), SHAM(darkgray bars) and ARTH(black bars) in comparison to SAL administration in NAIVE(white bars) animals. LIDO microinjection in the DMH of NAIVE and SHAM animals increased TF (A) and PF (B) latencies 10 and 20 minutes after its administration when compared to SAL. In ARTH animals, the tonic descending drive from the DMH is lost as LIDO failed to alter both TF and PW latencies. (SAL – saline; LIDO – lidocaine; ARTH – arthritis/arthritic animals; TF – tail-flick; PW – paw-withdrawal; PI – pre-injection; 10min – ten minutes after injection; 20min – twenty minutes after injection). (Mean ± SD) *p<0.05, **p<0.01, ***p<0.001.

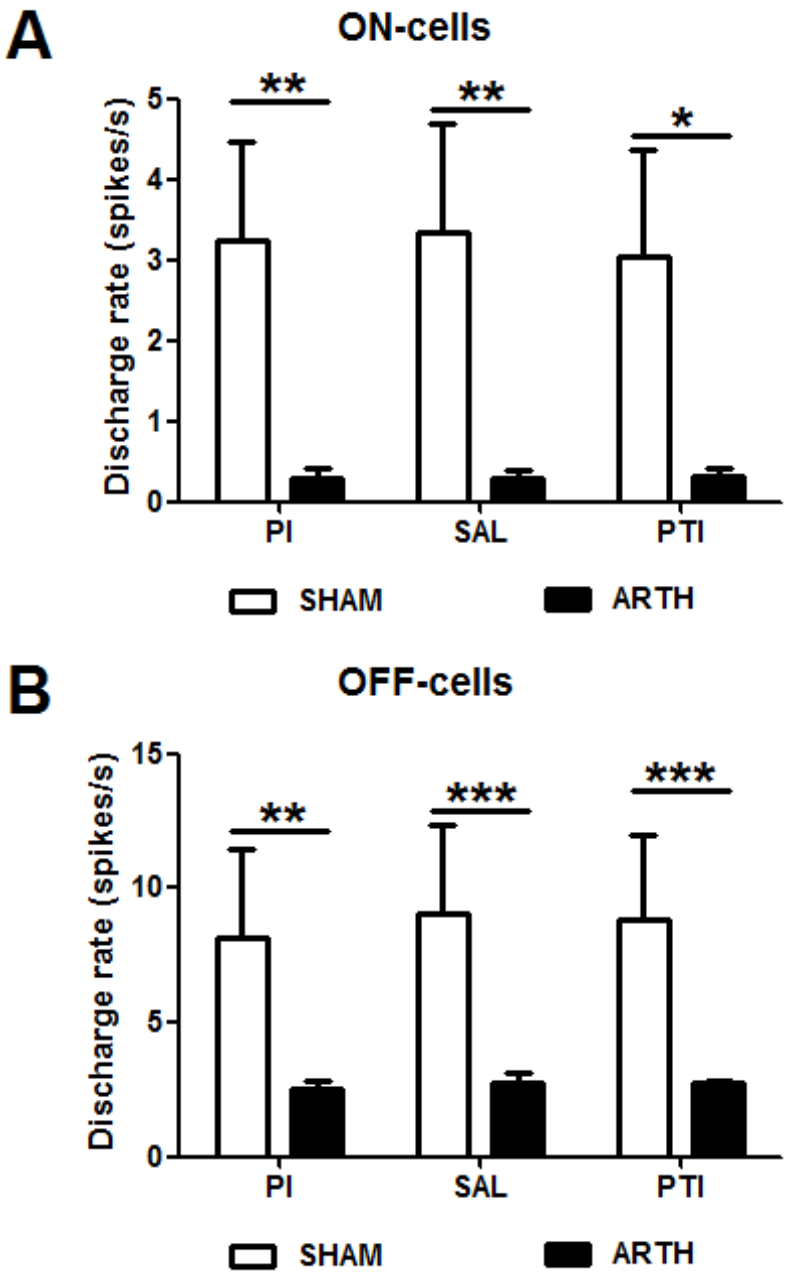

Figure 6 – The administration of SAL in the DMH had no effect upon spontaneous activity of ON- (A) and OFF-cells (B) of the RVM in both the SHAM and the ARTH groups. However it is important to note that the spontaneous activity of RVM neurones in SHAM and ARTH animals is significantly different. (A) The spontaneous firing rate of pronociceptive ON-cells of the RVM was significantly decreased in ARTH animals when compared to the SHAM group; (B) similarly, the spontaneous activity of antinociceptive RVM OFF-cells was significantly decreased in the ARTH group when compared to SHAM. (PI – pre-injection; I – injection; PTI – post-injection). (Mean ± SD) *p<0.05, **p<0.01, ***p<0.001

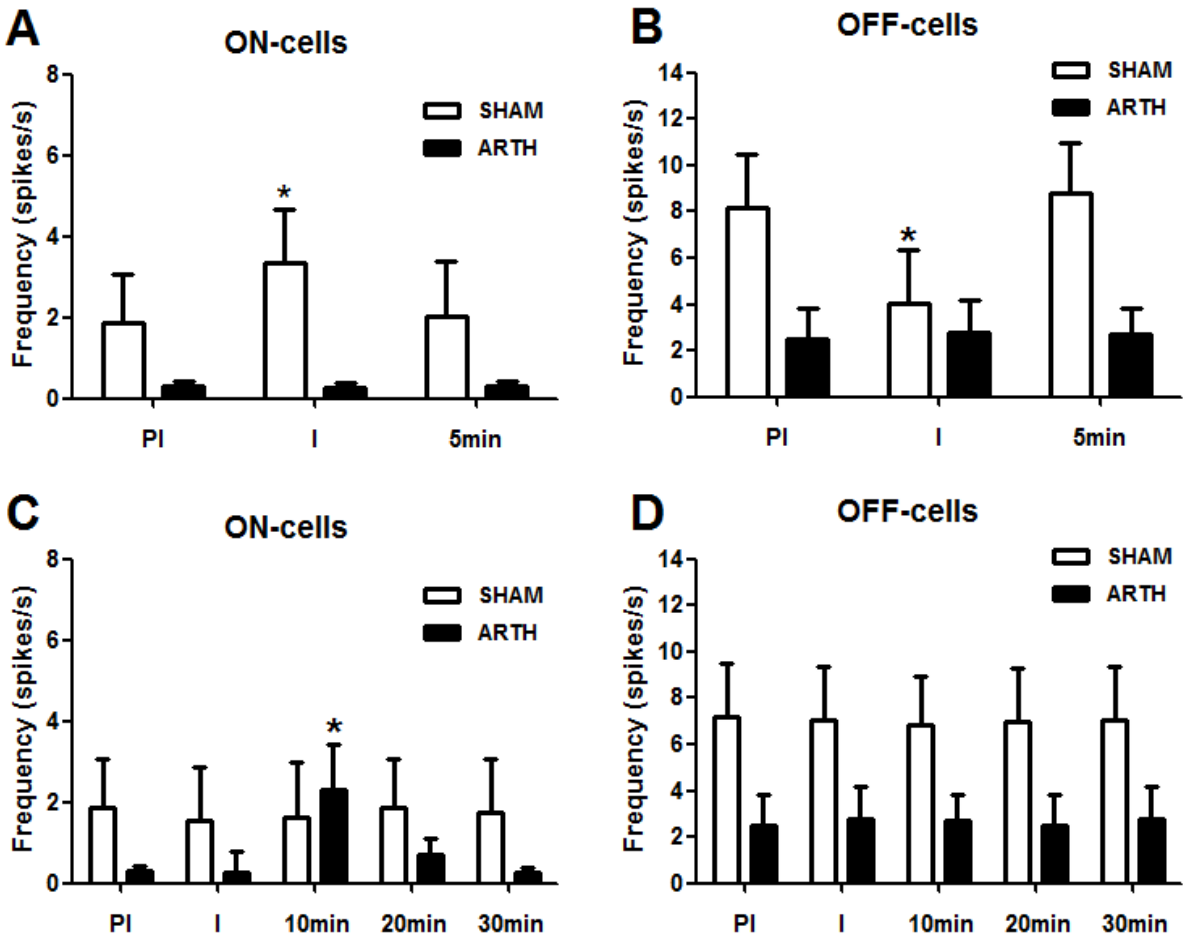

Figure 7 – Effect of the administration of GLU and LIDO in the DMH upon the spontaneous activity of RVM pain modulatory cells. Note that the administration of GLU in the DMH increased the spontaneous activity of RVM ON- (A) and decreased the spontaneous activity of OFF-cells (B) in SHAM animals, respectively, but had no effect in RVM neurones in ARTH animals. However when LIDO was microinjected in the DMH although it had no effect in the spontaneous activity of RVM cell in SHAM animals, it significantly increase ON-cell (but not of OFF-cell) spontaneous activity in ARTH animals. (PI – pre-injection; I – injection; 5min – five minutes; 10min – ten minutes after injection; 20min – twenty minutes after injection; 30min – thirty minutes after injections). (Mean ± SD) *p<0.05

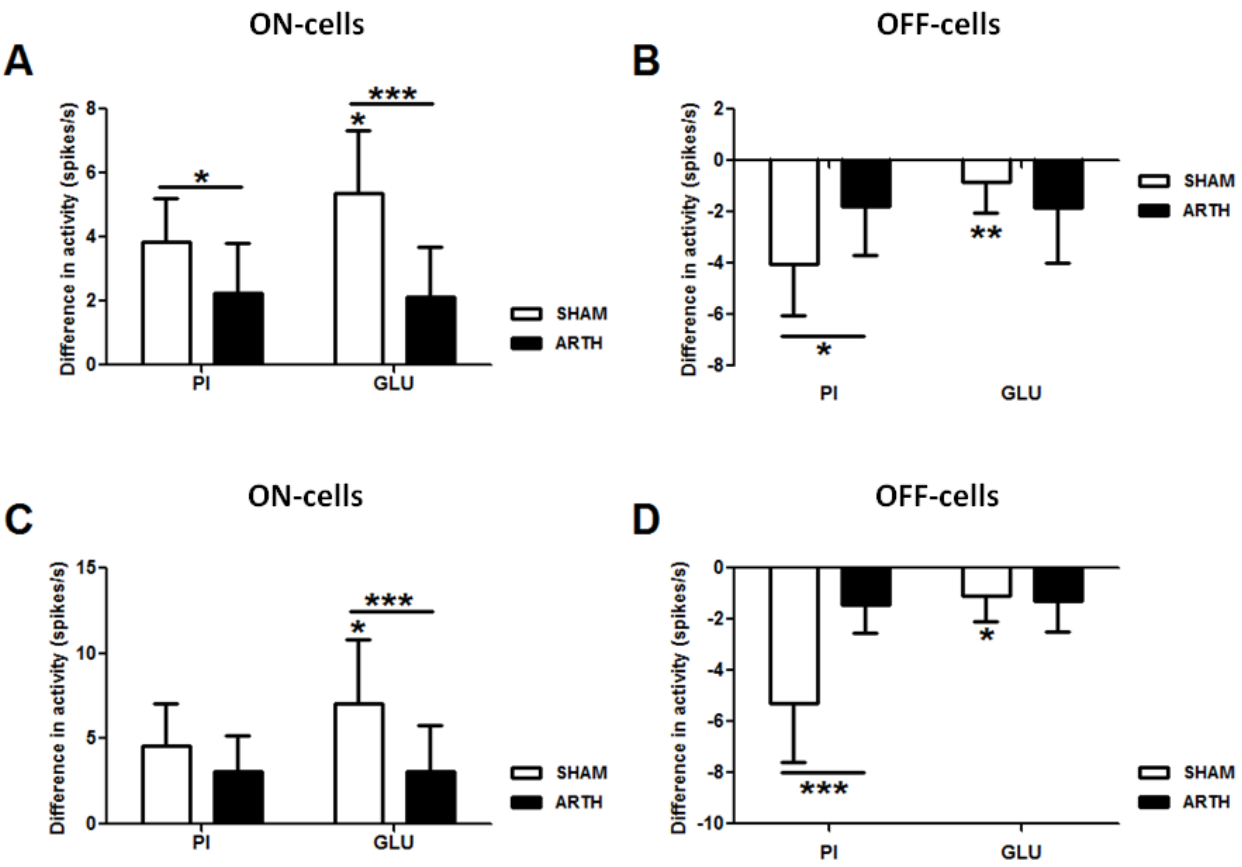

Figure 8 - Effect of the activation of the DMH by GLU upon the noxious-evoked activity of RVM ON- (A,C) and OFF-cells (B,D) in SHAM and ARTH animals. Note that GLU administration in the DMH of SHAM animals increased the response of RVM ON-cells during thermal (A) and mechanical (C) noxious stimulation, while this effect was absent in ARTH animals. The activity of RVM OFF-cells was decreased during thermal (B) and mechanical (D) noxious stimulation and this effect was again absent in ARTH animals, whose baseline response was already significantly decreased in comparison to SHAM. (PI – pre-injection; GLU – injection of GLU in the DMH). (Mean ± SD) *p<0.05, **p<0.01, ***p<0.001

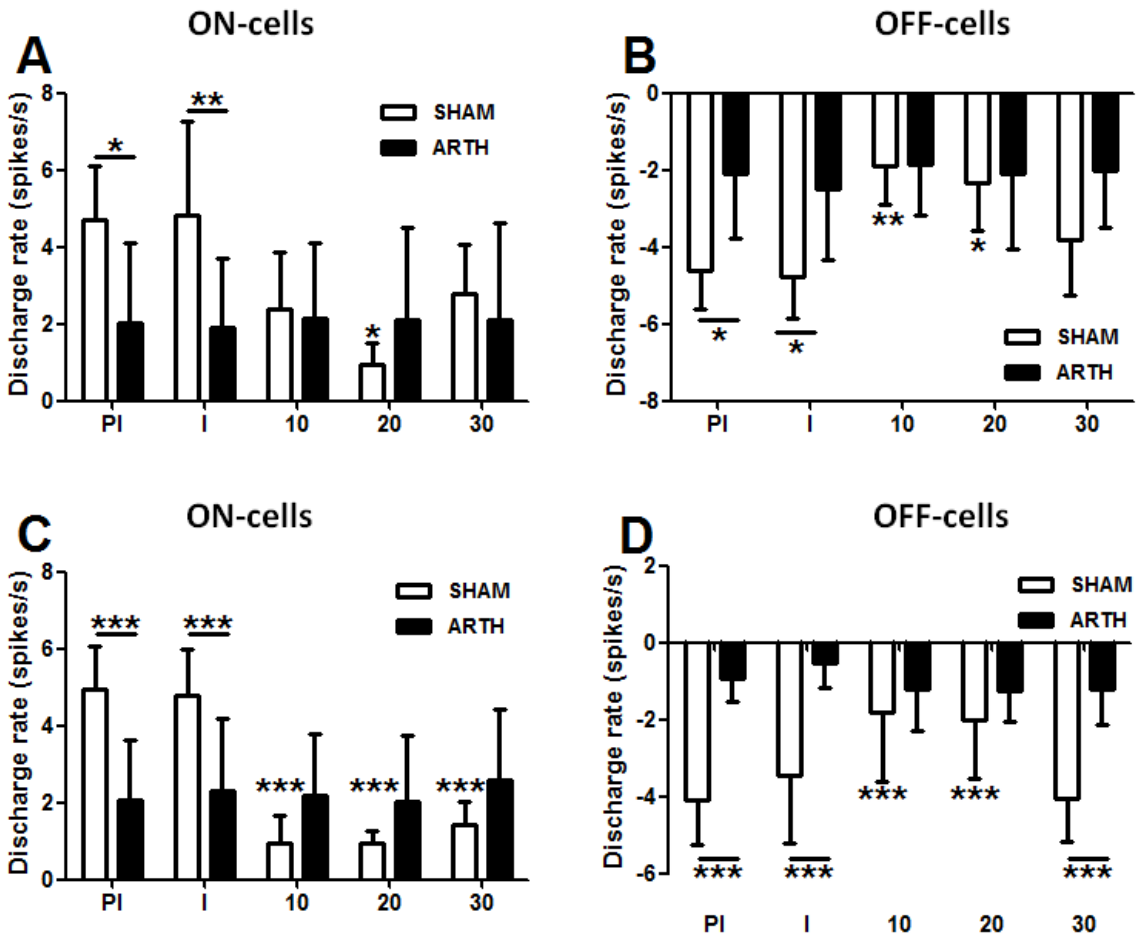

Figure 9 - Effect of the DMH inhibition by LIDO upon the noxious evoked activity of RVM ON- (A,C) and OFF-cells (B,D) in SHAM and ARTH animals. Note that LIDO administration in the DMH decreased the response of RVM ON-cells during thermal (A) and mechanical (C) noxious stimulation, while this effect was absent in ARTH animals. The activity of RVM OFF-cells was increased during thermal (B) and mechanical (D) noxious stimulation and this effect was again absent in ARTH animals, whose baseline response was already significantly decreased in comparison to SHAM. (PI – pre injection; I – injection; 10min – ten minutes after injection; 20min – twenty minutes after injection; 30min – thirty minutes after injections) (Mean ± SD) *p<0.05, **p<0.01, ***p<0.001

Chapter 3

Discussion

In this work, through the use of anatomical, behavioural and electrophysiological approaches, we sought to discriminate some of the underlying aspects of pain modulation by stress. We showed that a strong paradigm of chronic unpredictable stress (CUS) produced sustained analgesia after three weeks of treatment. By pharmacologically mimicking CUS, through the administration of corticosteroid agonists, we were able to correlate the resulting analgesia to changes in nociceptive neurotransmitter content in the spinal cord, to which each corticosteroid receptor contributed differently. A broad analysis of the neuronal network of the paraventricular nucleus of the hypothalamus (PVN), the effector of the stress response due to its role in the activation of the hypothalamic-pituitary-adrenals axis (HPA), highlighted the existence of afferent and efferent connections of this nucleus with several areas involved in the supraspinal pain control system. Pharmacological activation and inhibition of the PVN demonstrated that this area has an antinociceptive role that is exerted directly upon cells in brainstem areas classically involved in nociceptive modulation, such as the rostral ventromedial medulla (RVM) and the caudal ventrolateral medulla (CVLM). However, another counterpart of the stress response, the DMH, which appears to be implicated in SIH, exerts opposite pronociceptive effects by impinging in the same brainstem areas (RVM). In a chronic (arthritis) pain context, both the PVN and DMH descending drives upon nociception are altered due to neuronal plasticity resulting from the pain chronification process. In summary, this work provides detailed data on the alterations of nociceptive processing occurring during prolonged stress, its neuronal basis and the changes associated in a chronic pain context.

3.1. Animal models and experimental considerations

3.1.1. The choice of a stress model

The first objective of this work was to determine how nociception is modulated by chronic stress. When inducing chronic stress the choice of experimental protocol is the major challenge to achieve the desired goals, a sustained elevation of circulating corticosteroids while maintaining the sensitivity to stressors. In acute stress, a stressful event induces the activation of the HPA

axis and a surge in the release of corticosteroids (Conrad *et al.*, 2009). Corticosteroid secretion is under a negative feedback mechanism mediated by the binding of cortisol to specific receptors in the adrenal glands, the pituitary, the hypothalamus, the AMY and the hippocampus (Bradbury *et al.*, 1991; Conrad *et al.*, 2009). On the other hand, chronic stress is characterized by a hyperactivity of the HPA axis and subsequent sustained increase in the release of corticosteroids throughout time. While the acute stress response is aimed at restoring homeostasis, chronic stress (Swaab *et al.*, 2005), like chronic pain (Apkarian *et al.*, 2009), has no added biological value and when allowed to fully develop is highly detrimental resulting in neuronal dysfunction (Sousa *et al.*, 2008; Cerqueira *et al.*, 2008).

The most frequent sources of divergence between chronic stress-inducing protocols seem to be related to the duration, the order of application of the stressful event and the number of stressors involved. In terms of duration, acute experiments are usually designed to evaluate immediate cause-consequence events within a small time span (e.g. immobilization effect upon nociception). On the contrary, chronic stress paradigms have to be designed to mimic clinical settings in which the detrimental effects of stress develop mainly after two or three weeks of exposure to stressors (Herman, 2010). In rodents, the development of chronic stress is accompanied by the presence of biological markers that include: (i) in physical terms, a decreased weight gain and a decrease in grooming activities leading to a general deterioration in fur condition and the presence of bare skin patches; (ii) at the physiological level, an increase in glucose and lipid metabolism, suppressed neurogenesis and increased neuronal apoptosis (Bachis *et al.*, 2008), thymus atrophy and immunological impairment (Sapolsky *et al.*, 2000; Tannenbaum *et al.*, 2002). In our studies we opted for longer exposure periods of three to four weeks as most studies employing shorter periods failed to achieve a chronic stress phenotype (Vyas *et al.*, 2002).

Additionally, the sustained activation of the HPA axis requires that the stressful event applied is strong enough to avoid habituation. Several studies have demonstrated that the repeated application of the same stressor or of different stressors in the same order results in desensitization and a gradual decrease of the state of stress (Gamaro *et al.*, 1998; Rivat *et al.*, 2010). Through the use of unpredictable stimuli applied in a random order, animals develop a state of learned helplessness reinforcing the physiological stress response. The unpredictability factor is a key factor in CUS, as it suppresses the development of coping mechanisms in these animals (*Chapter 2.1.1*). Stress-induced actions have been attributed to glucocorticoid receptor

(GR) activation since in basal conditions the occupancy of these receptors is 10% when compared to 90% of mineralocorticoid receptors (MR). The exogenous administration of MR and/or GR agonists is often used to discriminate the contribution of these receptors to a physiological process. Importantly, this method has previously been proven to replicate the systemic effects of prolonged glucocorticoid secretion from the adrenal medulla, while animals display the typical biological markers of CUS (Sapolsky *et al.*, 2000). Hence, in the second set of experiments, we opted to pharmacologically mimic CUS through the exogenous administration of corticosterone (MR and GR receptor agonist) and dexamethasone (specific GR receptor agonist) to understand their involvement in the CUS-induced analgesia (*Chapter 2.1.2*).

In the last decades of clinical practice, corticoids have been extensively used in the treatment of inflammatory diseases, such as arthritis (Morand, 2007). Corticosteroid action within this context has been aimed mostly at the control of inflammation and the prevention of joint damage in order to avoid loss of function (Schett *et al.*, 2008) and not specifically as a pain management procedure. On the other hand, the typical pain syndromes that result from chronic pain conditions, amongst which arthritis, constitute constant discrete physiological stressors (Vierck *et al.*, 2010) that alter the normal HPA axis functioning and can contribute to the plastic changes in this system (Latremoliere and Woolf, 2009). Patients often report an increase in pain levels in the morning, which has been correlated to hypoactivity of the HPA axis and decreased levels of circulating cortisol (Cutolo *et al.*, 2005). Therefore, the second objective of this work was to determine how CUS impacted in the supraspinal pain control system (*Chapter 2.1.3*). Finally, after determining the neuronal connections of the paraventricular nucleus of the hypothalamus (PVN) (*Chapter 2.2.1*), we evaluated how the activation/inactivation of this area altered pain-like behaviour and neuronal activity in supraspinal pain control areas in the normal condition and in a pathological context (arthritis) (*Chapters 2.3.1 and 2.3.2*).

3.1.2. The choice of a pain-like model

Once more, the choice of an animal model representative of a persistent inflammatory component that replicate most processes observed in the human disease was crucial. The need to screen potential pharmaceutical agents has lead to the development of many models of arthritis (Bendele 2001), although the number of models available highlights the fact that none completely reflects all the features of the disease (Kaklamanis, 1992). Another difficulty is that arthritic conditions constitute a huge family of diseases amongst which the most common are

fibromyalgia, RA, psoriatic- and osteo-arthritis and gout. Human arthritis is characterized by the inflammation of joints leading to infiltration of granulocytes and later of mononuclear cells, synovial hyperplasia, bone erosion and formation, fibrosis and ankylosis of the joint (Roth et al., 2005). Some methods, like the injection of Complete Freund's adjuvant (CFA) in the rat's tail or paw, induce widespread effects that might result in polyarthritis (Decaris *et al.*, 1999), inappropriate for the study of specific mechanisms. Other protocols use the injection of carrageenan that induces the local release of neurotransmitters (Hong *et al.*, 2002), primary and secondary hyperalgesia as well as central sensitization (Urban and Gebhart, 1999), but its inflammatory process presents a small immune component (Guthrie *et al.*, 1996).

Alternatively, a method involving the unilateral microinjection of a mixture of carrageenan/kaolin in the knee joint (K/C arthritis) mimic human osteoarthritis while providing both a model of acute and chronic inflammation (Lawand *et al.*, 2000). Animals display ipsilateral heat hyperalgesia and mechanical allodynia only three hours after agent administration, which spreads contralaterally and is fully developed after seven days, lasting for at least eight weeks (Lawand *et al.*, 2000). Finally, this model complies with the criteria for drug screening as it allows predicting the efficacy of agents in humans, it is easily induced and replicated, and it displays a progressive development of a full spectrum of pathological features concomitant with the pathogenesis of the disease in humans (Lawand *et al.* 2000). Therefore, it seemed adequate to choose the kaolin/carrageenan monoarthritis model while studying the effect of PVN and dorsomedial hypothalamic nucleus (DMH) pharmacological manipulation upon the neuronal activity of areas implicated in supraspinal pain control (*Chapters 2.3.1, 2.3.2 and 2.3.3*).

3.1.3. Technical limitations

In the electrophysiological studies performed along this thesis two other limitations arose, the performance of acute neuronal recordings and the application of acute noxious stimuli to study a time-dependent process. The electrophysiological analysis followed the method developed for single cell extracellular recordings (Neugebauer and Li, 2003). This method presents, by itself, several limitations, the major of which is related to the fact that it is an acute recording technique applied at different time points in order to evaluate the effect of a dynamically adapting neuronal process. An alternative method would imply the chronic implantation of electrodes in supraspinal pain modulatory areas before the induction of arthritis and the continuous recording of cell activity throughout the experimental period in free moving animals (Silva *et al.*, 2008). Taking

into account that the descending modulation of pain is classically attributed to the net result of the activation/inactivation ratio of brainstem pronociceptive ON-cells and antinociceptive OFF-cells, instead of a single electrode recording it would be necessary to implant the electrodes and be able to record single-cells throughout the whole process of development of arthritis. Also limitative is the proximity needed between the electrode and the neurone in order to perform the recordings as any slight change in the coordinates on the electrode, as a consequence of free moving animal normal activity, could cause neuronal death and/or the loss of the cells being recorded. To overcome this difficulty, we opted to sample a large number of neurones at each time point, ensuring that the sampling was representative.

Joints, except for the cartilage, are innervated by Aβ, Aδ and C-fibres, allowing pain to be elicited through the application of noxious thermal, chemical and especially mechanical stimuli to the joints in conscious healthy humans (Dye *et al.* 1998). Electrophysiological studies have demonstrated the existence of two different sets of neurones that transmit noxious information from the joints to the spinal cord (Neugebauer *et al.* 1993), (i) a first set that converge noxious information from both the skin and deep tissues – mostly projecting to spinal wide-range-dynamic (WDR) neurones - and another set originating exclusively in deep tissues – mostly projecting to spinal nociceptive-specific (NS) neurones (Schaible *et al.*, 2009). In persistent inflammation, hyperalgesia and allodynia are the result of plastic changes within spinal pathways and descending pain modulatory systems that mediate joint pain. Hence, the use of phasic stimuli, like heat, cold and mechanical stimuli applied to the skin were chosen in order to activate the same spinal pathways responsible for transmitting the nociceptive information from the two sets of neurones innervating the joints, with colorectal distension being a visceral type of stimulation for further comparison. The drawback of this methodology is that we can detect alterations in evoked pain-like behaviours, but not on on-going (spontaneous) pain.

3.1.4. Behavioural assessment of pain

The biggest challenge of animal experiments involving pain perception is the lack of verbal communication. Hence, while the application of acute noxious stimuli results in the display of a series of standardized behavioural responses, its absence does not prove the animals are not feeling pain (LeBars *et al.*, 2001). The acknowledgement of these differences lead to the adaptation of the IASP definition of pain to animals as "an aversive sensory experience caused by actual or potential injury that elicits progressive motor and vegetative reactions, results in learned

avoidance behaviour, and may modify species specific behaviour, including social behaviour" (Zimmerman, 1986). Consequently, nociceptive behaviour is mainly ascertained by observing physical reactions of animals (van de Weerd *et al.*, 1994) to a stimulus known to cause pain to humans.

Throughout our studies, three different tests using noxious heat stimulation were used in order to overcome the limitations presented by each one alone. In the first work of this thesis, the effect of CUS upon nociception was evaluated weekly by stimulating the tail of the rat with radiant heat – tail-flick test (TF) – and determining the threshold of tail withdrawal from the heat source (D'Amour and Smith, 1941) (***Chapter 2.1.1***). As the TF measures mainly a spinal reflex (Sinclair *et al.*, 1988; Jensen and Yaksh, 1986; Mitchell and Hellon, 1977) and is prone to habituation (Carstens and Wilson, 1993), while evaluating the possible contribution of MR and GR receptors to the CUS-induced analgesia the hot-plate (HP) test was used simultaneously. The HP measures supraspinally integrated responses as animals placed in a pre-heated metallic plate (O'Callaghan and Holzman, 1975) display a series of behavioural responses - chaotic defensive movements (Knoll *et al.*, 1955), which include paw licking and jumping. Indeed the behavioural analysis highlighted the existence of both a spinal and supraspinally antinociceptive-mediated effect of CUS (***Chapter 2.1.2***).

In the following studies however, as we acquired a new apparatus, the HP was substituted by the paw-withdrawal test (PF) (***Chapters 2.3.1 and 2.3.3***). The PW test is identical in concept to the TF test, but presents some additional advantages: (i) the area of stimulation is the paw, which is not a thermoregulatory organ and hence less prone to bias, (ii) the animals move freely, thus without the additional stress of being restrained, (iii) this test was specifically developed in order to study hyperalgesia after inflammation (Hargreaves *et al.*, 1988), and (iv) the extent of the area of stimulation it smaller than when the HP is used, decreasing the chance of an involvement of the evoke diffuse noxious inhibitory controls (DNIC) (Le Bars *et al.*, 2001). Overall, the conjugation of these tests, in which the behavioural outputs are mediated by partially overlapping circuits, allowed a better tuning of the pain-like alterations triggered by CUS (per example, the correlation between pain-like behaviour differences and the neurochemistry of the dorsal horn after three or four weeks of corticosteroid treatment – ***Chapter 2.1.1***).

3.2. Chronic stress and pain modulation

Chronic pain and chronic stress are pathologies that share common features, mutually reinforcing their effects in numerous disorders. While a series of well characterized physiological and behavioural changes are at the base of the stress-induced analgesia present during the acute stress response (McEwen and Kalia, 2010), the effects of prolonged stress upon pain perception are not linear as bidirectional changes in nociception are observed (Satoh *et al.*, 1992; Quintero *et al.*, 2000). Our first aim was to demonstrate a clear correlation between the level of engagement of the HPA axis and pain-like behaviour **(Chapter 2.1.1)**.

Indeed, the young and old rats submitted to long-term application of a series of unpredictable physical stressors displayed sustained hypoalgesia (Pinto-Ribeiro *et al.*, 2004). As previously mentioned, our data conflicts with studies where chronic stress enhanced pain (Imbe *et al.*, 2006). A possible justification for this apparent paradox is the existence of distinctive descending modulatory pathways that are differently activated in response to homotypic (repeated single stressor) vs. heterotypic (variable stressor) stressors. Hyperalgesia, or enhanced pain-like behaviour, was observed in animals that were repeatedly exposed to the same stressor (Nasu *et al.*, 2010). This methodology leads to a gradual decrease of circulating ACTH and corticosterone levels, an evidence of the decline of HPA axis responsiveness (Harvey *et al.*, 2006). Remarkably, strong analgesic responses, in these animals, could be evoked if a novel stressor was introduced (Marti and Armario, 1998).

The stress response as an acute adaptive mechanism enables the individual to cope with challenges and to adjust to new situations. However, prolonged activation will desensitise this mechanism unless the intensity of the stimuli is able to overcome habituation. Taking this into consideration, the development of learned helplessness in our animals implies the involvement of an additional emotionally-dependent stressor. Thus it seems likely that, in the CUS model, the unpredictability of the stress factor evoked an additional psychological modulatory component (despair) to the equation (), positively reinforcing the stress response (sustained activation of the HPA axis) and contributing to prolonged analgesia. On the other hand, in clinical settings the opposite effect may be observed. Patients with chronic on-going pain (a source of repeated identical stressor) suffer HPA axis hyporeactivity and present increased anxiety and depression (Cutolo *et al.*, 2005), whereas patients suffering from mood disorders often suffer from HPA axis dysfunction and persistent pain (Fishbain, 1997). The possibility of neuronal remodelling of the

areas directly involved in pain modulation could not be discarded as it has been demonstrated that chronic stress promotes important structural changes in several brain areas associated to memory and cognition (Dias-Ferreira *et al.*, 2010; Romeo, 2010; Cerqueira *et al.*, 2008). In line with this reasoning, two other experiments were designed to evaluate the presence of sustained chronic stress-mediated functional changes in prime areas of the descending pain modulatory circuitry, such as the rostral ventromedial medulla (RVM) **(Chapter 2.1.3)** and the dorsal horn of the spinal cord **(Chapter 2.1.2)**.

Our results show that, in the brainstem, sustained HPA axis activation altered the neuronal activity of the RVM while it also impaired nociceptive transmission in the superficial spinal dorsal horn. The RVM is considered an end point of supraspinal pain modulation (Heinricher *et al.*, 2009; Almeida *et al.*, 2006; Pertovaara and Almeida, 2006), integrating inputs from the hypothalamus, the limbic system, cortex and brainstem areas such as the midbrain periaqueductal gray (PAG) (Hermann *et al.*, 1997). Additionally, The RVM modulates spinal nociceptive transmission by direct descending projections from presumptive pain modulatory neurones, the pronociceptive ON-cells and antinociceptive OFF-cells (Fields *et al.*, 1995; Neugebauer *et al.*, 2009). Our results demonstrate a significant decrease in the number pain modulatory ON- and OFF-cells active in CUS animals pointing to a loss of functionality in the RVM (***Chapter 2.1.3***). Additionally, although the spontaneous activity of both ON- and OFF-cells was augmented, preventing us from ascertaining a possible descending net effect, the noxious-evoked activity of pronociceptive ON-cells was significantly inhibited while no changes were observed in antinociceptive OFF-cells. These data are in accordance with the behavioural analgesia observed, as a partial loss of RVM function associated with impaired pain facilitation would inhibit nociception (***Chapter 2.1.3***). Curiously, selective loss of cellular activity in the RVM has previously been described to occur during inflammation (Montagne-Clavel and Oliveras, 1994), a pathology strongly associated with HPA axis dysfunction. Additionally, it is possible that the RVM cells undergo dendritic atrophy in response to overwhelming stress-induced input, similar to what was already observed in hippocampal neurones (Cerqueira *et al.*, 2008). Further stereological and morphological studies in this area would help to understand the impact of CUS at the cellular level in the supraspinal pain control system.

In parallel to the chronic stress-mediated RVM impairment, our data also demonstrates a strong involvement of GR receptors upon spinal nociceptive transmission and pain-like behaviour, as the

analgesia observed in the third week for CORT-treated animals (where MR and GR are activated) was mimicked by DEX administration (specific GR activation) (*Chapter 2.1.2)*. Concomitantly, in the DEX-treated group, in the third and fourth weeks of treatment, GR activation also resulted in a decrease in the expression of spinal nociceptive neurotransmitters (CGRP and SP) and an increase in the availability of $GABA_{B2}$ receptors. The GR-mediated effect was not completely unexpected since not only GRs have a greater basal availability, when compared to MRs, but the analysis of GR/MR receptor distribution throughout the rat brain already showed a stronger expression of GRs in brainstem areas such as the PAG and the RVM (Kolber *et al.*, 2008) while MR is strongly expressed in the limbic system (McEwen *et al.*, 1968). In addition, in the spinal cord, the terminals of nociceptors that express SP and CGRP, which are involved the transmission of noxious input from the periphery to the spinal cord, also co-express GR (DeLeon *et al.*, 1994).

However, in the fourth week of pharmacological treatment (DEX and CORT), the dichotomy between the recovery in the nociceptive processing of the CORT-treated group and the prolonged analgesia in the group treated with DEX was quite intriguing (*Chapter 2.1.2*). CORT-treated animals, in which a simultaneous activation of both MR and GR receptors is maintained, displayed a full recovery in nociception, measured by the TF and HP tests. This was further supported by a recovery to basal expression levels of nociceptive transmitter (CGRP) in the spinal cord. On the other hand, exclusive GR activation (DEX treatment) maintained the increased withdrawal threshold in the TF and HP, which was accompanied by a decreased spinal expression of SP and CGRP. Two main factors appear to contribute to this discrepancy between CORT and DEX after four weeks of treatment: the co-activation of MR in addition to GR in the recovery of the CORT-group, and the stronger deregulation of pain modulatory circuitries in DEX-treated animals (*Chapter 2.1.2*). In fact, it has been demonstrated that although much less available in basal conditions, MR activation is essential to the development of behavioural plasticity and recovery (Kolber *et al.*, 2008). Furthermore, Berger and colleagues (2006) had already demonstrated that mice where the MR gene had been inactivated in adulthood, exhibited a normal anxiety-like behaviour, but lacked behavioral flexibility, which could contribute to our contrasting CORT vs. DEX behavioural results. In summary, this study attributes an essential role to the equilibrium between MR/GR activation in long-term adaptation of pain circuits to chronic stress, identical to what is observed for emotional behaviour (Rozeboom *et al.*, 2007). This author showed that a chronic mild elevation in the activation of MRs in the forebrain decreased

anxiety-like behaviour in mice through the modulation of the expression of GRs and serotonin receptors in the hippocampus, although MR and GR appear to identically regulate the activity of the HPA axis, the ratio between MR/GR is significant to the mechanisms involved in emotional reactivity.

Alternatively, if we hypothetically transpose the peripheral effects of GR activation upon CGRP expression in primary afferents (decreased expression) to a more central action, the divergence between adaptative/maladaptive changes could partly be a consequence of changes in supraspinal CGRP content. CGRP expression has been described in the hypothalamus (Dhillo *et al.*, 2003), where it is able to activate the HPA axis (Kovacs *et al.*, 1995) and it is also present in the limbic system (van Rossum *et al.*, 1997), namely in the central nucleus of the AMY, where it has been proven to exert a facilitatory role (Han *et al*, 2010). Therefore, in DEX- but not CORT-treated animals, the reduction of CGRP in the AMY would contribute to a decrease pain-like behaviour.

3.3. Role of the PVN in descending nociceptive modulation in normal and arthritic animals

The ability of corticosteroids to modulate pain-like behaviours is a clear evidence of the involvement of the HPA axis in mediating the effects of chronic stress upon nociception. However, a specific role of the paraventricular nucleus of the hypothalamus (PVN) upon nociception and in pathologies that affect HPA axis function remained to be demonstrated. Through the use of retrograde and anterograde anatomical tracers (*Chapter 2.2.1*) it was possible to highlight the major input and output areas of the PVN and to identify those that are known to participate in pain control. The PVN shares intense bidireccional projections with many other hypothalamic nuclei, such as the dorsomedial and the arcuate nuclei, and the caudal hypothalamus besides strong inputs from the limbic system, notably the amygdala, which is in accordance with previous specific anatomical studies. Within the midbrain and the brainstem, numerous pain modulatory areas project to the PVN, such as the periaqueductal gray matter (PAG) (Farkas *et al.*, 1998), the nucleus tractus solitarius (NTS) (Sawchenco and Swanson, 1982), the rostral ventrolateral medulla (RVLM) (Card *et al.*, 2006), the dorsal reticular nucleus (DRt) (Leite-Almeida *et al.*, 2006) and locus coeruleus (LC) (Reyes *et al.*, 2005). On the other hand, the PVN projects massively to the PAG followed by the NTS, and, more modestly, to the RVM and the CVLM (*Chapter 2.2.1*). Although most of these targets have already been revealed

by previous studies (Herman *et al.*, 1997; Pyner and Coote, 2000; Almeida *et al.*, 2006; Geerling *et al.*, 2010), the present work shows the overall map of PVN projections.

Taking into account the above anatomical data and that CUS decreased the spontaneous and evoked responses of RVM pronociceptive ON-cells (*Chapter 2.1.3*), then it is possible that the PVN may play a role in this process. Therefore, through pharmacological manipulation, the nociceptive regulatory effect of the PVN upon the spinal dorsal horn and on the response properties of RVM cells was electrophysiologically assessed in normal animals. Additionally, a second set of animals with monoarthritis (ARTH) was also used in order to evaluate potential changes in the descending pain-modulatory drive from the PVN (*Chapter 2.3.1*), as this pathology is known to alter the neurochemistry of this nucleus (Shanks *et al.*, 1998) and chronic stress also changes the PVN neurochemistry (Flak *et al.*, 2009). Our results show that the descending drive from the PVN is exerted upon RVM pain modulatory cells and spinal neurones. A phasic and tonic antinociceptive drive upon pain-like behavioural responses mediated by serotonergic and noradrenergic (but not opioidergic) descending projections from the PVN is observed in controls animals, but not in the arthritic group. Accordingly, the PVN-induced antinociceptive drive relayed by RVM cells in naive animals is also reduced in monoarthritis.

The fact that modulation of spinal transmission by the PVN was mediated by serotonergic and noradrenergic pathways substantiates a role for the RVM and the LC in mediating this effect, as these nuclei are the source of spinal serotonin (Fields and Basbaum, 1999) and noradrenaline (Basbaum and Fields, 1999; Westlund *et al.*, 1982), respectively. Moreover, Aimone and colleagues (1987) demonstrated that the analgesia produced by electrical stimulation of either the PAG or the RVM is mediated by serotonergic and/or noradrenergic - but not by opioid - receptors in the spinal cord (like the descending drive from the PVN). The loss of the descending serotonergic and noradrenergic drive from the PVN after the induction of arthritis points to a possible remodelling of the pain circuitry. Aira and colleagues (2010) reported, in chronic pain, the occurrence of spinal serotonergic receptor subtype-specific changes that were at the origin of enhanced spinal hyperexcitability, and may counteract the descending antinociceptive drive from the PVN. Additionally, Wei and colleagues (2010) demonstrated that, during the development of persistent pain, RVM descending serotonergic spinal projections contribute to pain facilitation. On the other hand, the tonic noradrenergic regulation of nociception has a protective role in disease, functioning as an antihyperalgesic mechanism (Mansikka *et al.*, 2004) in response to peripheral

carrageenan inflammation (Tsuruoka and Willis, 1996). The decrease in descending noradrenergic PVN drive is probably related to plastic changes in spinal neurones and not to a decrease in noradrenaline expression as it has been demonstrated that, in supraspinal areas, its concentration doubled after arthritis (Harbuz *et al.*, 1994).

In the RVM, the spontaneous activity of both ON- and OFF-cells is increased in arthritis (***Chapter 2.3.1***). Additionally, RVM NEUTRAL-cells have been described as serotonergic (Wei *et al.*, 2010) and are possibly recruited in chronic pain conditions (Miki *et al.*, 2002). Hence, the increase in RVM ON- and OFF-cell activity after the settlement of arthritis might reflect the recruitment of former "silent" pain modulatory neurones due to changes in the neurochemistry of the RVM. On the other hand, the effect of the PVN upon RVM nociceptive cells differs between normal and arthritic conditions, although always contributing to an overall antinociceptive role. These dissimilar activation patterns, before and after the induction of arthritis, are not random, as the PVN inhibits RVM ON-cells in control animals and facilitates OFF-cells in arthritic animals (***Chapter 2.3.1***). If we consider that the magnitude of the response of ON-cells to noxious input will dictate the intensity of the evoked-behavioural response (Jinks et al., 2007), then a major control of the PVN upon RVM cell activation is more important in "normal" conditions. Contrarily, taking into account that RVM OFF-cells are able to activate more OFF-cells and to inhibit surrounding ON-cells, then the enhancement of the activity of these cells by the PVN in arthritis may reveal a tentative counterbalance of the ascending nociceptive drive resulting from ARTH.

The PVN projects also to the CVLM (***Chapter 2.2.1***) (Geerling *et al.*, 2010), an area strongly implicated in descending pain modulation (Tavares and Lima, 2002), and thus may mediate also the descending modulatory action from the PVN. Additionally, we demonstrated for the first time the presence of CVLM pronociceptive (+) cells and antinociceptive (-) cells (***Chapter 2.3.2***), as already documented in other supraspinal pain control areas, as the PAG (Heinricher *et al.*, 1987) and the RVM (Heinricher *et al.*, 2009). In arthritic animals, nociceptive activity of the CVLM appears to partly mediate the antinociceptive drive from the PVN, but through a different mechanism of that triggered by the PVN upon the RVM (***Chapter 2.3.1***). Contrary to the PVN-RVM circuitry, the activation of the PVN significantly decreased presumptive CVLM ON-cell evoked-response during all modalities of peripheral noxious stimulation. Since the effect of the PVN upon both the RVM and the CVLM in arthritic animals points to an increased antinociceptive modulation, it is possible that a fine tuning of the descending modulatory action from the PVN is

dependent on the balance of the antinociception mediated by both nuclei. Future studies should evaluate the interaction of these PVN-medullary pathways in coping with the development of chronic (arthritic) pain.

3.4. Role of the DMH in descending nociceptive modulation in normal and arthritic animals

Stress is known for long to produce analgesia (SIA) (Butler and Finn, 2009), but acute and chronic stress also produce hyperalgesia (SIH) (Imbe *et al.*, 2006). It is already established that the DMH gives rise to autonomic, endocrine and behavioural changes associated with stress, such as increased circulating levels of ACTH, increased heart rate and blood pressure and hyperventilation. Additionally, inactivation of the DMH interferes with autonomic and neuroendocrine responses, validating the physiological relevance of the DMH in stress response. However, while the PVN has been associated with SIA (Filaterov *et al.*, 1996), the DMH has been attributed a role in SIH, achieved through a strong activation of ON-cells and suppression of OFF-cells following DMH desinhibition (Martensen *et al.*, 2009).

By direct manipulation of the DMH, we demonstrated that in normal pain-free conditions this nucleus has a clear pronociceptive role that is mediated, at least in part, by both an increase of RVM ON- and a decrease of RVM OFF-cells (***Chapter 2.3.3***). Again, and like previously discussed for the PVN-RVM and PVN-CVLM circuits (see above), the variation in the balance of the activity of RVM nociceptive cells is used to give an anatomical mechanism and support to the alterations observed in pain behaviour. This rationale, in addition to the simple logic of analyzing the variation in the activity of ON- and OFF-cells, is supported by elegant studies performed by Heinricher and colleagues. In fact, they showed that neurotensin applied to the RVM was hyperalgesic at low doses and analgesic at higher doses and that these pain behaviours were associated to a selective activation of ON-cells and a recruitment of OFF-cells, respectively (Neubert *et al.*, 2004). Additionally, the secondary thermal hyperalgesia resulting from topical application of mustard oil to the skin was associated with an increase in the activity of ON-cells as well as a decrease in the activity of OFF-cells (Kincaid *et al.*, 2005). Taking all these data into account, we can include the hypothalamic DMH in the up-to-now restricted group of areas of the neuroaxis with a clear descending pronociceptive effect upon nociception, as shown previously for the DRt (Almeida *et al.*, 1996; 1999; reviewed by Lima and Almeida, 2002; Zhang *et al.*, 2005; Almeida *et al.*, 2006; Sotgiu *et al.*, 2008). Nonetheless, the parameters that led to a stress-related hyperalgesic, rather than analgesic, response are not yet defined. It is possible that

this outcome is triggered by forebrain pathways that integrate the motivational-affective dimensions of pain (Thompson and Swanson, 1998). Unexpectedly, the descending pronociceptive role of the DMH is lost in arthritic animals (*Chapter 2.3.3*), which was confirmed by the absence of a DMH descending drive upon the RVM nociceptive cells in the same conditions. This indicates that the DMH may play a role in acute stress-induced hyperalgesia and in the facilitation of withdrawal responses to stimuli threatening the integrity of the organism. However, and contrary to the PVN, the DMH has apparently no role in the increased noxious-evoked nociceptive drive that occurs in chronic (arthritic) pain. Nonetheless, future studies should evaluate a potential role for the DMH in the spontaneous pain that is associated with the development of arthritis.

Chronic nociceptive pain, as a result of arthritis, alters the PVN modulated endogenous pain control system, both at the spinal and supaspinal levels. As the PVN is the mediator of the physiologic response to stress, the deregulation of its descending drive upon the brainstem constitutes a substrate for the association between chronic stress and chronic pain states. This hypothesis is further supported by the neuroplasticity of the pain modulatory RVM cells observed after the administration of corticosteroids.

3.5. References

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Chapter 4

Conclusions and Future Perspectives

During the course of our studies we were able to identify some of the underlying mechanisms that contribute to analgesia in animals under chronic unpredictable stress, while evaluating at the same time the influence of the nuclei responsible for mediating the stress response upon the neuronal activity of areas of the pain control system. More specifically we demonstrated that:

1. The analgesic effect of chronic unpredictable stress (CUS) is the result of profound plastic physiological and neurochemical changes both in the spinal cord and in supraspinal areas modulating nociception.

2. The CUS-evoked plastic changes appear to be mediated mainly by the activation of GR receptors, but the equilibrium between the activation of both MR and GR corticosteroid receptors appears to be essential for the animals to maintain the ability to adapt to the experimental challenges.

3. The direct effect of CUS upon the activity of RVM pain modulatory cells was restricted to neurones receiving solely nociceptive inputs, as the activity of RVM convergent neurones that also processed innocuous stimulation were not altered. In these cells, CUS resulted in a decrease of both the spontaneous and noxious-evoked activity of RVM pronociceptive ON-cells, which is in accordance with the behavioural antinociception observed.

4. Through the use of anatomical tract-tracing techniques, the PVN connections with the brain showed that it is part of an extensive network comprising many supraspinal pain modulatory areas. These included regions involved not only in the processing of the different dimensions of the pain experience, but also in descending pain modulation.

5. The PVN, the effector of the stress response, decreases tonic and phasically spinal nociceptive transmission through serotonergic and noradrenergic (but opioidergic) pathways, while modulating also the activity of pain modulatory neurones in the RVM

and CVLM. The loss of serotonergic and noradrenergic descending drive, as well as the change in the ability of the PVN to modulate RVM and CVLM cell response in arthritic animals, indicates that the PVN-mediated antinociceptive systems suffer profound remodelling in pathological conditions.

6. Arthritis induces substantial changes in the basal and evoked activity of pain modulatory neurones in both the RVM and CVLM. Interestingly, although an overall activation of basal neuronal activity in both nuclei is observed, subtle changes in the noxious-evoked responses of these cells in each nucleus will occur depending on stimulus modality applied. This indicates the possibility of a specific contribution of each area towards the enhancement of a specific component of chronic pain.

7. SIH shares common descending modulatory pathways with SIA, through the modulatory effect of the DMH upon the activity of pain modulatory cells in the RVM. Surprisingly, the circuitry centered in the DMH and mediating noxious-evoked hyperalgesia does not participate in the development of arthritis.

Further studies soughing to address the interaction between these two systems, chronic pain and chronic stress should focus on:

1. Assessing the specific contribution of PVN outputs to nociceptive processing in a prolonged stress scenario, as it was demonstrated that the persistent HPA activation by arthritis altered not only the activity of pain modulatory areas, but also impaired PVN-mediated antinociception.

2. Determining the time-dependent biochemical and physiological changes occuring in prime areas within the neuronal network meditating pain chronification in arthritis. An initial behavioural assessment of cognitive performance would help to identify possible limbic and cortical counterparts involved in inflammatory pain. Additionally, and since the enhancement or inhibition of a specific area during persistent pain states is a dynamic mechanism, the prolonged recording of the neuronal activity in these areas in association to pain modulatory centres would help to uncover critical time points in the pain chronification process.

3. Investigating possible structural changes associated with the development of chronic inflammatory pain. The stereological analysis of the structure of the PVN and areas involved in pain descending modulation would highlight changes occurring in the number of neurones and glial cells as well as in the total volume of these areas. In parallel, the morphological study of the dendritic trees would further contribute to shed some light upon the dynamics of inflammatory pain at the cellular level.